Production, purification and characterization of Coxsackievirus B1 virus-like particles

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Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Viruksen kaltaiset partikkelit (VLP:t) aiheuttavat useimmiten laajan ja vahvan immuunivasteen elimistössä, joten ne sopivat hyvin rokotekehityksen kohteiksi. Tämän työn tavoitteena oli tuottaa VLP:ta coxsackievirus B1:lle (CVB1). Tavoitteina oli myös optimoida tuotto hyönteissoluissa ja löytää skaalautuva puhdistusmenetelmä CVB1-VLP:lle. Coxsackie B (CVB) virukset aiheuttavat vaikeita sairauksia ja kuolemantapauksia, sekä terveydenhuollolle suuria kustannuksia maailmanlaajuisesti, mutta CVB viruksille ei ole hoitoja tai rokotteita.

Tutkimusmenetelmät: CVB1-VLP:ta tuotettiin bakulovirus-hyönteissolu ekspressio systeemillä. Parhaimman tuottotason saavuttamiseksi, vertailtiin eri hyönteissolulinjoja, flashBAC DNA variantteja, bakulovirusinfektio-pitoisuuksia ja -aikoja, sekä kasvatusmediumeja ja kasvatustilavuutta. VLP:t konsentroitiin joko tangentiaali flow filtraatiolla tai PEG-presipitaatiolla ja puhdistettiin monivaiheisella ioninvaihtokromatografialla (IEX). Puhdistettujen VLP:ien laatua ja puhtautta arvioitiin SDS-PAGE:lla, Western blottauksella, dynaamisella valonsironnalla ja elektronimikroskopialla.

Tutkimustulokset: Paras tuottotaso saavutettiin High Five hyönteissoluilla ja ne ekspressoivat kaksi kertaa enemmän ekstrasellulaarista VLP:tä kuin Sf9 solut. FlashBAC ULTRA ja flashBAC GOLD ekspressoivat huomattavasti enemmän ekstrasellulaarista VLP:tä kuin flashBAC ja flashBAC PRIME, eikä ensin mainittujen tuottotasoissa ollut merkittävää eroa High Five soluissa. Paras tuottotaso saavutettiin viruspitoisuudella MOI 1 50 ml:n kasvatustilavuudessa, eikä kasvatustilavuutta onnistuttu skaalaamaan tehokkaasti ylös. Ultrasentrifuugauksella onnistuttiin tuottamaan 67 % puhdasta VLP:tä kun taas IEX:illä 100 % puhdasta. Saanto 100 % puhtaalle VLP:lle oli 0.6 mg/l.

Johtopäätökset: Korkein CVB1-VLP tuottotaso saavutettiin High Five hyönteissoluilla käyttämällä 5 päivän infektiota, 50 ml:n kasvatustilavuutta, joko flashBAC GOLDia tai ULTRA:aa ja MOI-arvoa 1. 100 % puhdasta CVB1-VLP:tä tuotettiin käyttämällä kolmivaiheista IEX:iä.

Avainsanat: Coxsackievirus B1, CVB1, Viruksenkaltainen partikkeli, VLP, hyönteissolu, ioninvaihtokromatografia, rokote

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Abstract

Background and aims: Virus-like particles (VLPs) are promising vaccine candidates, usually eliciting broad and strong immune responses in humans. The aim of this study was to produce VLPs for coxsackievirus B1 (CVB1). CVB viruses cause severe morbidity and mortality worldwide, causing a major impact on the health care system, but there are no treatments or vaccines available against CVBs. The specific aims of this study were to find out the most efficient insect cell-line for CVB1-VLP production, and finding a scalable purification method for CVB1-VLP and finally the characterization of CVB1-VLP.

Methods: Baculovirus-insect cell expression system was used in the production of CVB1-VLPs. Various steps in the CVB1-VLP production were optimized including the type of insect cell line and the flashBAC DNA variant, the Multiplicity Of Infection (MOI) used for CVB1-VLP amplification, baculovirus cultivation time, type of cell growth medium and culture volumes and the CVB1-VLP purification method. VLPs were concentrated either using tangential flow filtration or PEG-precipitation and purified using multi-step ion exchange chromatography (IEX) purification. The quality and the purity of the CVB1-VLPs were estimated with SDS-PAGE, Western Blotting, dynamic light scattering and Transmission Electron Microscopy analyzes.

Results: The highest CVB1-VLP production level was obtained in High Five cells and they expressed two times more extracellular VLP than Sf9 cells. FlashBAC ULTRA and flashBAC GOLD expressed considerably more extracellular VLP than flashBAC or flashBAC PRIME in High Five cells. Further, the production levels of flashBAC ULTRA and flashBAC GOLD were almost equal. MOI 1 seemed to give the highest production levels. Highest yield was in 50 ml culture and culture volume could not be efficiently scaled up. 67 % pure VLP was produced with ultracentrifugation, while 100 % pure VLP was produced with IEX. Yield for 100 % pure VLP was 0.6 mg/l.

Conclusion: Highest VLP yield was achieved with High Five insect cells using 5 days baculovirus infection, 50 ml culture volume, flashBAC GOLD or ULTRA and MOI 1. 100 % pure CVB1-VLP was generated using three step IEX.

Key words: Coxsackievirus B1, CVB1, Virus-like particle, VLP, insect cell, ion exchange chromatography, vaccine

Preface

The work reported in this Master's thesis has been carried out at Faculty of Medicine and Life Sciences in the University of Tampere between August 2016 and April 2017.

I would like to thank my group leader Associate Professor Vesa Hytönen for giving me an opportunity to work in Protein Dynamics group and my supervisor Ph.D Minna Hankaniemi for providing me guidance with this project. I also want to thank Merja Jokinen for providing me practical guidance to work with insect cells. I am also thankful to Ulla Kiiskinen and Niklas Kähkönen for your excellent technical assistance. I would also like to thank other members of the Protein Dynamics and THERDIAB groups: Olli Laitinen, Juha Määttä, Rolle Rahikainen, Azizi Latilef, Niila Saarinen and Soili Lehtonen, for collegian support, friendship and sharing their knowledge whenever I needed it. Thanks for your trust in me and encouragement throughout my time at Faculty of Medicine and Life Sciences institute.

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Contents

1 Introduction **2**

2 Review of the Literature **3**
2.1 Enteroviruses (EVs) . . . 3
2.1.1 An overview of enteroviruses (EVs) . . . 3
2.1.2 Enterovirus Virions and Viral Genome Organization . . . 4
2.2 Virus-like particles (VLPs) . . . 5
2.2.1 Characteristics of VLPs . . . 5
2.2.2 VLP production systems . . . 6
2.2.3 Downstream processing of VLPs . . . 7
2.3 Vaccines . . . 7
2.3.1 A short introduction to vaccines . . . 7
2.3.2 Vaccines against viral diseases . . . 9

3 Aims of the Study **14**

4 Materials and Methods **15**
4.1 Generating baculovirus expressing CVB1-VLP . . . 15
4.1.1 Design and construction of the CVB1-VLP transfer vector . . . 15
4.1.2 Cell culture and generation of the VLP-producing recombinant baculovirus . . . 15
4.1.3 Infectivity titration of the recombinant virus . . . 17
4.2 Optimizing the production of CVB1-VLP in insect cells . . . 17
4.3 Purification of CVB1-VLPs . . . 17
4.3.1 Clarification . . . 17
4.3.2 Concentration and buffer exchange . . . 18
4.3.3 Purification and polishing . . . 19
4.4 Characterization of CVB1-VLPs . . . 20
4.4.1 SDS-PAGE and Western Blotting . . . 20
4.4.2 Total protein concentrations . . . 20
4.4.3 Dynamic light scattering . . . 20
4.4.4 Transmission electron microscopy . . . 21

5 Results **22**
5.1 Production of VLPs . . . 22
5.1.1 Optimization of CVB1-VLP production in insect cells . . . 22
5.1.2 CVB1-VLP purifications . . . 26
5.2 Biophysical characterization of VLPs . . . 32
5.2.1 The purified VLPs show correct size and morphology . . . 32

6 Discussion **34**
6.1 Evaluation of results . . . 34
6.1.1 Optimizing VLP productions . . . 35
6.1.2 CVB1-VLP purification . . . 36

7 Conclusions **37**

8 References **38**

9 Appendixes **41**
9.1 LacZ control vector 41

List of Abbreviations

AHC	hemorrhagic conjunctivitis
CHIKV	chikungunya virus
CMV	cytomegalovirus
CVA	coxsackievirus A
CVB	coxsackievirus B
Da	Dalton
DAMPs	damage associated molecular patterns
DCs	dendritic cells
DF	diafiltration
DLS	dynamic light scattering
DNA	deoxyribonucleic acid
dpi	days post infection
EV	enterovirus
HFMD	hand-foot-and-mouth disease
High Five	*Trichoplusia ni*
HIC	hydrophobic interaction chromatography
HPV	human papillomavirus
IEX	ion exchange chromatography
MOI	multiplicity of infection
NoV	norovirus
PAMPs	pathogen-associated molecular patterns
PCR	polymerase chain reaction
PdI	polydispersity index
PEG	polyethylene glycol
Polh	*Polyhedrin*
PRRs	pattern-recognition receptors
PV	poliovirus
RNA	ribonucleic acid
RT	room temperature
RV	rhinovirus
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel
SEC	size-exclusion chromatography
Sf	*Spodoptera frugiperda*
SOP	Standard Operating Procedure
T1D	type 1 diabetes
$TCID_{50}$	50 % tissue culture infective dose
TEM	transmission electron microscopy
TFF	tangential flow filtration
UTR	untranslated region
VLP	Virus-like particle
VP	viral protein
VPg	virus encoded protein
WB	Western blotting

1 Introduction

Enteroviruses (EV) are the most common viruses affecting humans. Enteroviruses cause a wide range of diseases including common cold, febrile illness, otitis media, meningitis, encephalitis, myocarditis, hand, foot and mouth disease, polio, paralysis, pancreatitis and severe life-threatening infections in newborns [1]. In addition, enterovirus infections have been connected to the development of chronic diseases such as chronic cardiomyopathies, chronic fatigue syndrome, type 1 diabetes (T1D) and asthma [2].

Alongside traditional poliovirus vaccines, EV vaccines have been developed against EV71 and two of them have recently received regulatory approval in China [3]. However, preventive applications against other EVs, would lessen the burden of these diseases. Vaccines are among the greatest achievements of modern medicine and vaccination has probably made a greater impact on global health than any other intervention in medicine.

Non-infective virus-like particles (VLPs) are empty particles comprised of viral structural proteins. Thanks to the preservation of virus structure, VLPs can elicit broad and strong immune responses and are promising vaccine candidates. The majority of VLPs are produced using the baculovirus-insect cell expression system, which involves the infection of insect cells with a recombinant baculovirus that drives the foreign gene expression [4].

Previously, in this research group, VLPs for norovirus (NoV) [5] and coxackievirus B3 (CVB3) [6] have been produced using baculovirus-insect cell expression system. Previously, the recombinant baculovirus for CVB3-VLP was generated using Bac-to-bac baculovirus expression system [6]. In this study, the recombinant baculovirus was generated using flashBac baculovirus expression system [7], which has been shown to enhance VLP yield for EV71 [8].

The aim of this study was to produce and characterize virus-like particles for coxsackievirus B1 (CVB1), and optimize CVB1-VLP production. Different insect-cell lines (Sf9, Sf21 and High Five), flashBAC DNA variants (flashBAC, flashBAC ULTRA, flashBAC GOLD and flashBAC PRIME), MOI-values for CVB1-VLP amplification, baculovirus infection time, type of cell growth medium and culture volumes and the CVB1-VLP purification method were compared. Titers of different virus stocks were determined using end-point dilution method. VLPs were concentrated either using tangential flow filtration or PEG-precipitation and purified using multi-step ion exchange chromatography. The quality and purity of the VLPs was estimated using SDS-PAGE, Western blotting (WB), dynamic light scattering (DLS) and transmission electron microscopy (TEM).

2 Review of the Literature

2.1 Enteroviruses (EVs)

2.1.1 An overview of enteroviruses (EVs)

Enteroviruses (EV) belong to the one of the largest virus families, *Picornaviridae*. *Picornaviridae* family can be classified into 29 genera and enterovirus is one of these. EVs have been classified into 12 species, including EVs A-D, RV A-C, and five EV species that only infect animals (EV-E to EV-J) [9]. These viruses include coxsackie A and B viruses, echoviruses, polioviruses (PVs), numbered EVs, and rhinoviruses (RV) [9].

EVs are transmitted via the fecal-oral route or via respiratory transmission, depending on the type [9]. EVs have two primary replication sites, the gastrointestinal tract and the respiratory tract, from where the virus can spread to the target organs via the blood circulation [9].

The Global Polio Eradication Initiative was launched in 1988 by the World Health Assembly with oral poliovirus vaccine (OPV) [10] and due to this wide vaccination program, PV has nearly become extinct nowadays, but nevertheless, the virus remains endemic in three countries (Afghanistan, Nigeria and Pakistan) and sporadic PV outbreaks occur [9]. Before development of vaccine, polio was one of the most feared illness in the world, because it killed or paralyzed more than half a million people a year [11].

Genotype EV71 is responsible for the outbreaks of hand-foot-and-mouth disease (HFMD) in the Asia-Pacific region since the late 1990s [8, 9, 10]. The largest epidemic occurred in China, causing 7.2 million cases of HFMD and claiming 2457 lives from 2008 to 2012 [8, 10]. EV71 affects mostly children causing several neurological complications and even death [8, 9]. In the case of EV71, the error-prone RNA polymerase is responsible for high rates of mutability and rapid antigenic divergence, especially in the immunodominant surface VP1 protein, leading to immune escape [10].

EV-D68, which was discovered in 1962 [12], has recently drawn attention because of an outbreak in the United States and to a smaller extent in the rest of the world [9]. EV-D68 causes respiratory illness and likely spreads from person to person when an infected person coughs, sneezes, or touches a surface that is then touched by others. This virus has been also suspected as the cause of a rare polio-like paralysis [12].

RVs are the major cause of the common cold [9]. They can infect both the upper and the lower respiratory tract [9]. RVs can cause severe pneumonia in the elderly and immunocompromised patients, as well as exacerbations of chronic obstructive pulmonary disease and asthma [9, 13].

Coxsackieviruses are divided to A and B groups based on early observations of their

pathogenicity in mice [14]. CVAs were noted to cause a flaccid paralysis, which was caused by generalized myositis, while CVBs were noted to cause a spastic paralysis due to focal muscle injury and degeneration of neuronal tissue [14]. CVAs infect the skin and mucous membranes, causing herpangina, acute hemorrhagic conjunctivitis (AHC), and hand-foot-and-mouth (HFM) disease [14]. CVBs infect the heart, pleura, pancreas, and liver, causing pleurodynia, myocarditis, pericarditis and hepatitis [14]. Both group viruses can cause nonspecific febrile illnesses, rashes, upper respiratory tract disease, and aseptic meningitis [14]. Lately, CVBs has been connected to type 1 diabetes (T1D) [15].

2.1.2 Enterovirus Virions and Viral Genome Organization

Picornaviruses are small positive-strand RNA viruses with genome of about 7.5 kb in length [9, 16]. The genome is encapsidated by an icosahedral capsid, forming a virion of around 30 nm in size without an envelope [9]. The viral capsid is composed of 60 identical units (protomers) each consisting of the four structural proteins VP1-VP4 [16]. Enterovirus genome consists of a single open reading frame that contains P1, P2 and P3 regions (Figure 1). P2 and P3 regions encode nonstructural proteins responsible for virus replication and virulence while P1 region encodes P1 precursor that can be cleaved by 3CD protease into VP0 (further cleaved to give VP4 and VP2 during viral maturation), VP1 and VP3. These three proteins spontaneously assemble into icosahedral procapsid and pack the RNA genome into the provirion [6, 17]. Size of CVB capsid proteins are 37 kDa (VP0), 31 kDa (VP1), 29 kDa (VP2), 26 kDa (VP3) and 7.5 kDa (VP4) [6].

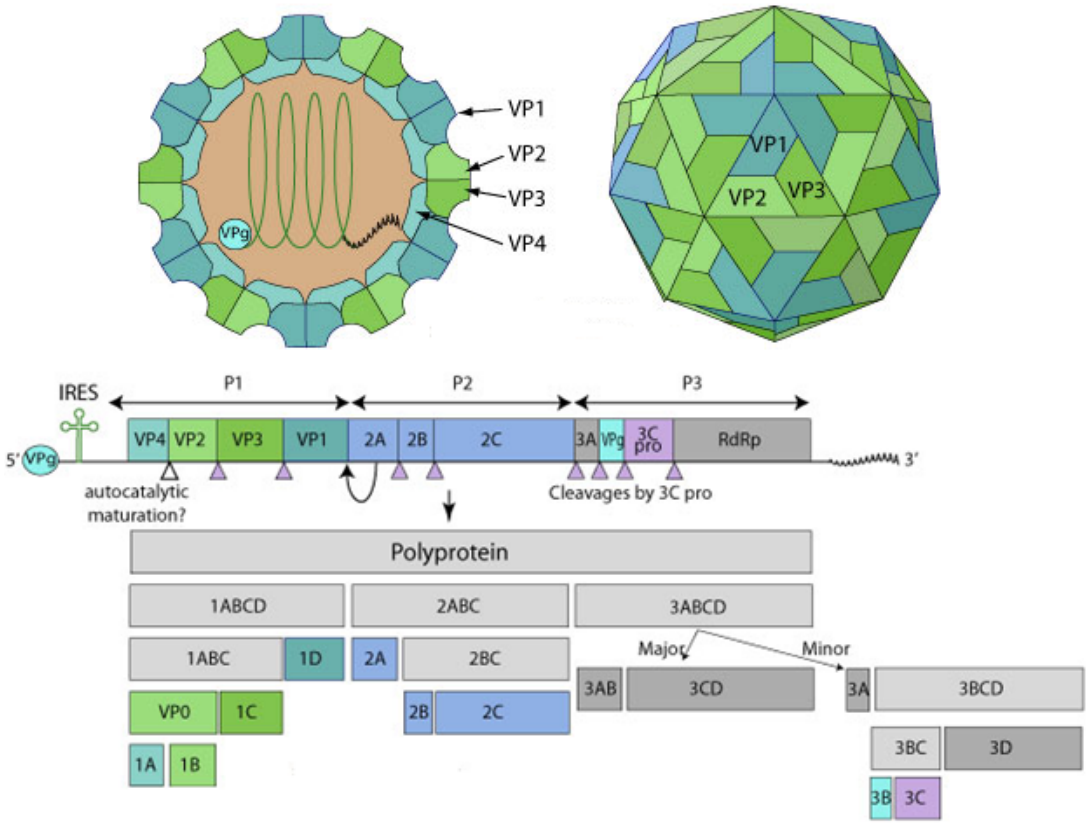

Figure 1: ***Genome organization and capsid structure of enterovirus.*** *All the structural proteins are encoded by the P1 region of the genome. The P2 and P3 regions encode seven non-structural proteins 2A-2C and 3A-3D. At the 5'-and 3'-end the genome contains untranslated regions (UTR), which are highly structured. Vpg = virus encoded protein. The figure is modified from Solomon et al. 2010 [18].*

2.2 Virus-like particles (VLPs)

2.2.1 Characteristics of VLPs

VLPs consist of protein shells (virus surface structures), but they lack the core genetic material making them non-infectious and unable to replicate and are therefore useful e.g. as vaccines [4]. The basic studies of virus structure and assembly led to the observation that when expressed by using recombinant expression systems, many viral structural proteins intrinsically self-assemble to VLPs. This observation led to better immunological mimics of whole-virus particles and led to a renaissance in subunit vaccine development [19]. Since the beginning of the 1980s, over 100 VLPs have been produced and characterized and derived from 35 different virus families [20]. These VLP based-vaccines combines many of the advantages of whole-virus-based and recombinant subunit vaccines, exhibiting a high safety profile. VLPs can stimulate strong B and T-cell immune responses and have been shown to exhibit self-adjuvanting abilities. These characteristics have made

VLPs attractive stand-alone vaccine candidates for many viral diseases [19].

VLPs are supramolecular assemblies with a well-defined geometry, usually icosahedrons or rod-like structures, and diameters in the range of 20-120 nm [19]. VLPs can be classified depending on the original virus taxonomy or depending on the synthesis method (e.g. animal, yeast, plant or cell-free expression). Another classification of VLPs relies on their architecture and distinguishes enveloped and non-enveloped VLPs, as well as native or chimeric VLPs. Non-enveloped VLPs are typically composed of one or more viral structural proteins, whereas enveloped VLPs consist of the host cell membrane with viral proteins displayed on the outer surface [21].

2.2.2 VLP production systems

There are many expression systems for the production of VLPs. In this study, the baculovirus-insect cell expression system (using Sf9, Sf21 or High Five cells) was used. However, various species of yeast (e.g. *Saccharomyces cerevisiae*), bacteria (e.g. *E. coli*), various mammalian cell lines (e.g. *Vero*), plants (e.g. tobacco) and cell-free systems are used for the production of VLPs. Recombinant expression systems are based on viral vectors: baculovirus, poxviruses, alphavirus replicons, etc. [19]. All production systems have their own advantages and drawbacks.

Animal cells are the most suitable expression system for VLPs, that require post-translational modifications. However, mammalian cell expression system has some limitations like high production costs, potential safety concerns, and difficulties with scaling up production. Instead, insect cell expression system is less expensive, easier to scale up and can be used for the simultaneous expression of many proteins and they also have the ability to post-translationally process and modify recombinant proteins. They can be also cultured without supplements derived from mammalian organisms, which lower the risk of contaminations [21]. Due to these reasons, most VLPs are produced by using the baculovirus/insect cell expression system, which involves the infection of insect cells with a recombinant baculovirus that drives the foreign gene expression by the very late strong promoters *polyhedrin (polh)* or *p10*. Traditionally, Sf9 insect cell is most widely utilized host cell, but High Five insect cell line has also gained popularity [4].

Yeasts are well-established for the expression of recombinant proteins, but they differ from mammalian cells in their post-translational modification of expressed proteins, particularly protein glycosylation pattern. Lately, several studies have demonstrated successful self-assembly of VLPs in *Pichia pastoris* or *Saccharomyces cerevisiae* cells [22, 23]. Another option for VLP synthesis is plant expression systems, which is cost-effective, scalable, and free of mammalian pathogens. The most widely used expression system for

recombinant proteins are bacteria's [21].

2.2.3 Downstream processing of VLPs

Purification process of VLPs may consist of many steps, such as clarification, concentration and buffer exchange, primary purification, polishing and sterile filtration steps. The first purification step depends on whether the VLPs are released to the extracellular medium. If the VLP is not efficiently secreted, a cell lysis or other extraction step might be required before the actual clarification step. The clarification step is used to remove cell debris and large aggregates from harvested VLP. This step is usually done by using centrifugation or/and depth filtration (with 0.45 and 0.2 µm filters) [24]. Clarified VLP is then concentrated e.g. with diafiltration (DF), tangential flow filtration (TFF) [24] or polyethylene glycol (PEG) precipitation [4, 6] to decrease the volume of the extract. Buffer exchange can be done e.g. by using TFF or diafiltration (DF) [4, 24]. Primary purification step is used to reduce DNA and endotoxin levels. This step is usually done by using affinity or ion exchange chromatography [24]. Some VLPs are also purified by ultracentrifugation with CsCl or sucrose gradient [8, 25]. Polishing step is used to remove any remaining contaminants and can be done by using size-exclusion (SEC) or other type chromatography [24, 26]. As in any other biopharmaceutical process, sterile filtration is typically the final step before vialing [24].

2.3 Vaccines

2.3.1 A short introduction to vaccines

A vaccine is a harmless variant or derivative of a pathogen that stimulates the immune system to mount defenses against the harmful pathogen. Active immunity can develop from the introduction of antigens into the body through immunization, often called vaccination. The virus causing cowpox, a mild disease usually seen in cows, was used over two centuries ago as the first vaccine (from the Latin *vacca*, cow). Vaccination with cowpox was significant because it enhanced the immune response to the closely related and far more dangerous smallpox virus. Today many sources of antigens are used to make vaccines, including inactivated bacterial toxins, killed microbes, weakened microbes and even genes encoding microbial proteins [27]. See Figure 2. In the case of smallpox, a vaccine was available long before viruses were known as causing agents [28]. Figure 3 shows when different viruses were discovered and vaccines against them were developed.

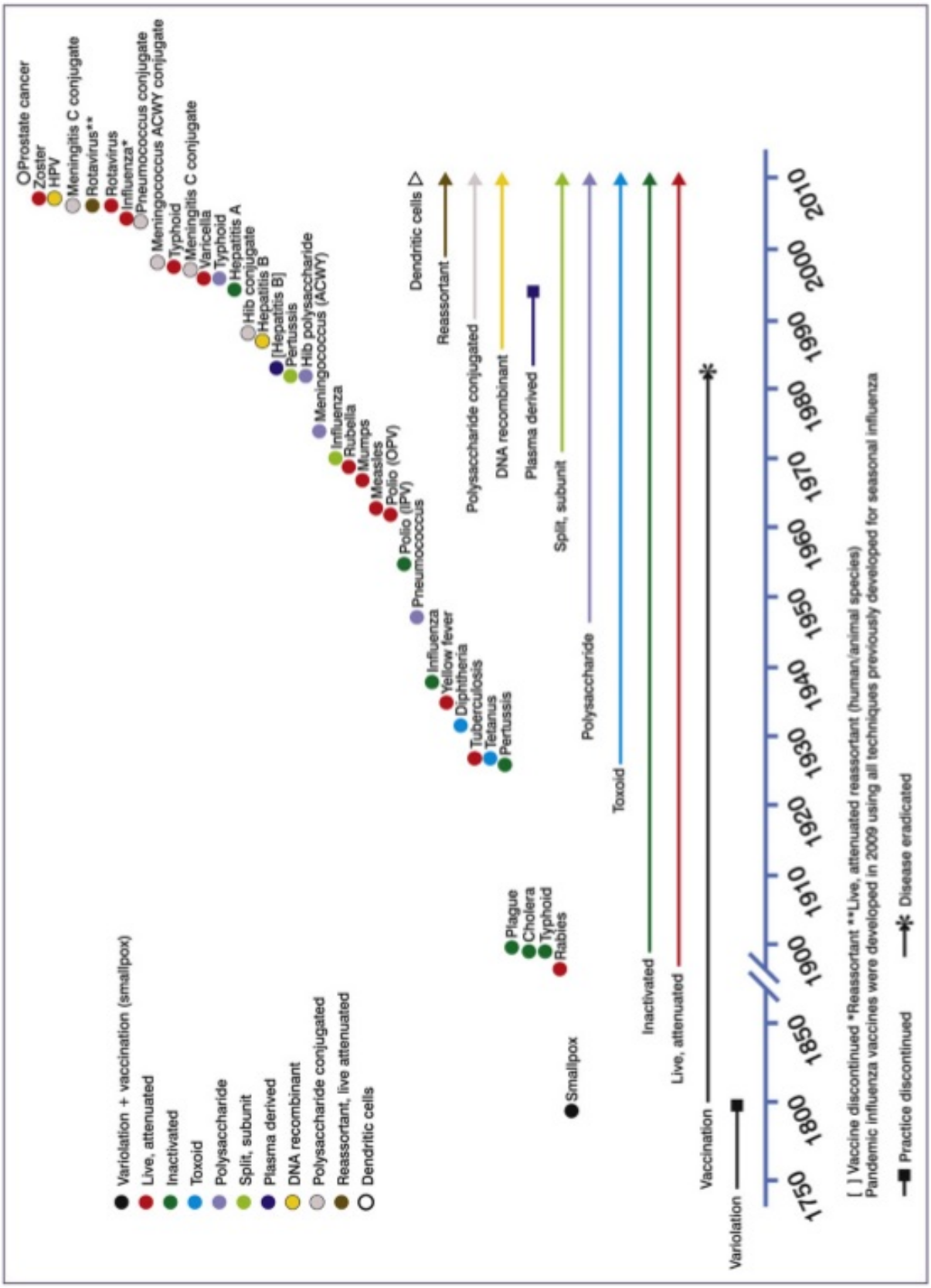

Figure 2: ***Vaccines and technologies.*** *Vaccine development timeline from the first practice of variolation. Figure is modified from Bonanni & Santos, 2011 [28].*

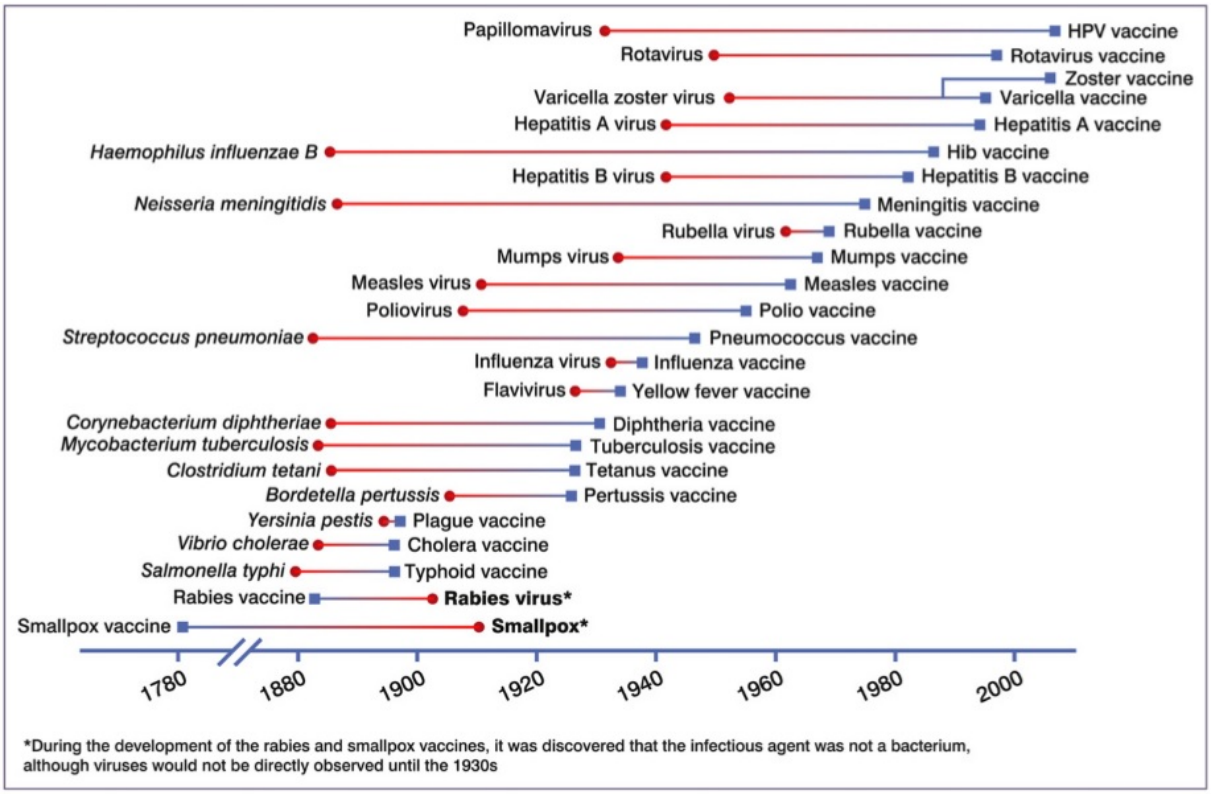

Figure 3: ***Pathogen isolation and vaccines.*** *Depending on availability of appropriate technology, there may be considerable variations in time between pathogen identification (sphere) and development of vaccine (box). Figure is modified from Bonanni & Santos, 2011 [28].*

> *"Vaccines have made the second most major contribution to the control and eradication of infectious diseases after the distribution of clean water"* [28].

2.3.2 Vaccines against viral diseases

Vaccines are generally classified as (variolation (smallpox),) live attenuated (viral or bacterial) vaccines (rabies, tuberculosis, yellow fever, polio, rotavirus, etc.) or inactivated vaccines that include inactivated whole organisms (typhoid, cholera, influenza, hepatitis A, etc.), toxoids (diphtheria, tetanus, etc.), protein subunit vaccines (influenza, anthrax, rabies, etc.) and virus-like particles (human papillomavirus (HPV) and chikungunya virus (CHIKV), future vaccine), purified bacterial polysaccharides (pneumococcus, meningococcus, typhoid, etc.) and their conjugate vaccines (Hib conjugate, meningitis C conjugate, etc.), DNA vaccines (hepatitis B and HPV) and dendritic cell vaccines (prostate cancer) (Figure 4) [28, 29, 30].

Most of the current vaccines are still produced by reducing the pathogen's virulence. These include biological attenuation of virulence, e.g. by repeated passage of viruses in cell-culture or embryonated eggs, and chemical inactivation of whole micro-organisms.

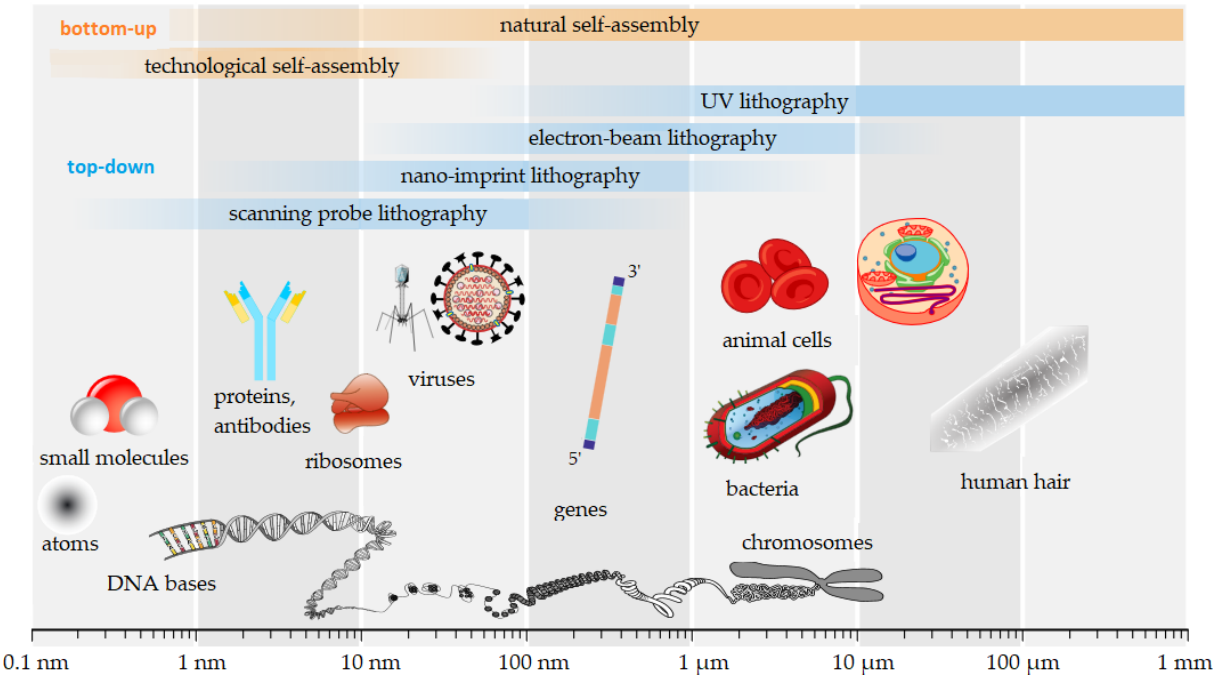

Figure 4: ***The size scales of various molecules contained in vaccines and other biological assemblies***. *Figure is modified from Paumier et al. 2009 [31].*

These classical vaccines induce efficient and prolonged immunity due to their ability to mobilize both humoral (B cells) and cellular (T cells) immune response (Figure 5). However, use of the classical vaccines present relevant drawbacks regarding safety and other issues. There is a risk of incomplete inactivation, which can lead to spreading of the disease the vaccine was intended to prevent. For this reason, live-attenuated vaccines are contraindicated for use in newborns, expectant mothers and immunocompromised individuals [19].

Instead of the entire microbe, subunit vaccines include only the antigens or purified single proteins that best stimulate the immune system. Subunit vaccines suffer from poor immunogenicity, because of incorrect folding and small size [32]. Instead, virus-like particles (VLPs), which are a specific class of subunit vaccines, can elicit wide and strong immune response and are promising vaccine candidates [4]. These VLPs are empty particles comprised of viral structural proteins. They lack the core genetic material making them non-infectious and safe to use [4].

DNA vaccines are one example of vector-based vaccines that are currently in development. One of their major advantage is their ability to induce the local expression of target antigens and subsequently elicit T_H1-biased and $CD8^+$T cell responses along with T_H1-biased antibody production (See Figure 6). In DNA vaccination, the genes for a microbe's antigens are introduced into the body and some cells will take up that DNA and start

to make the antigen molecules. The cells secrete the antigens and display them on their surfaces. In other words, the body's own cells become vaccine-making factories, creating the antigens necessary to stimulate the immune system. However, DNA vaccines tend to display low immunogenicity in humans and this has hindered their development [33]. Many different strategies have been tested to address this problem, including use of more efficient promoters and codon optimization, addition of traditional or genetic adjuvants, electroporation, intradermal delivery and various prime-boost strategies [34, 35].

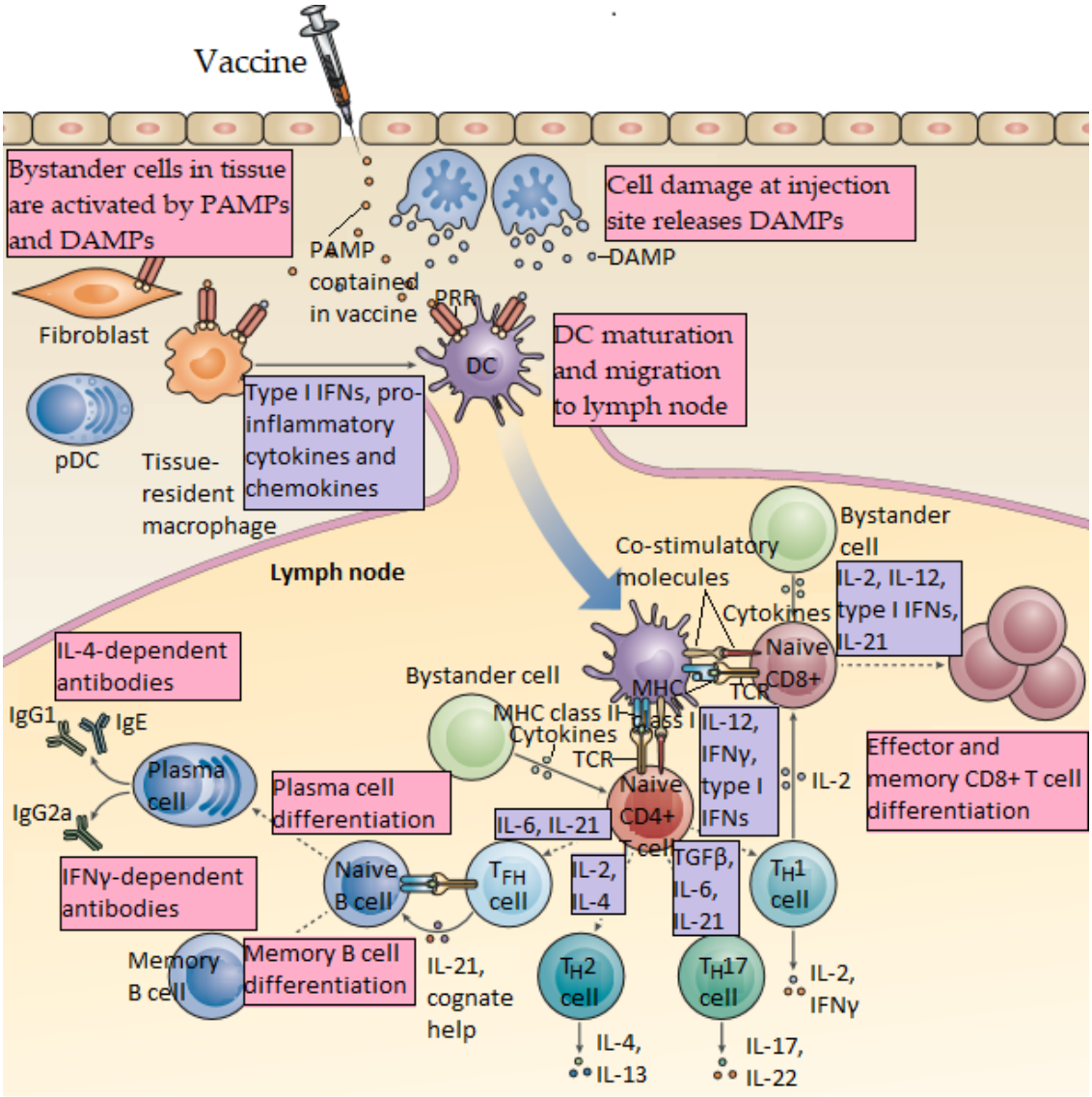

Figure 5: ***Induction of adaptive immune responses to vaccines through PRR-mediated dendritic cell activation.*** *Vaccines may contain pathogen-associated molecular patterns (PAMPs) or may induce the local release of damage-associated molecular patterns (DAMPs). These PAMPs and DAMPs are detected directly by pattern-recognition receptors (PRRs) expressed by dendritic cells (DCs), leading to DC activation, maturation and migration to the lymph nodes. Figure is modified from Desmet & Ishii, 2012 [33].*

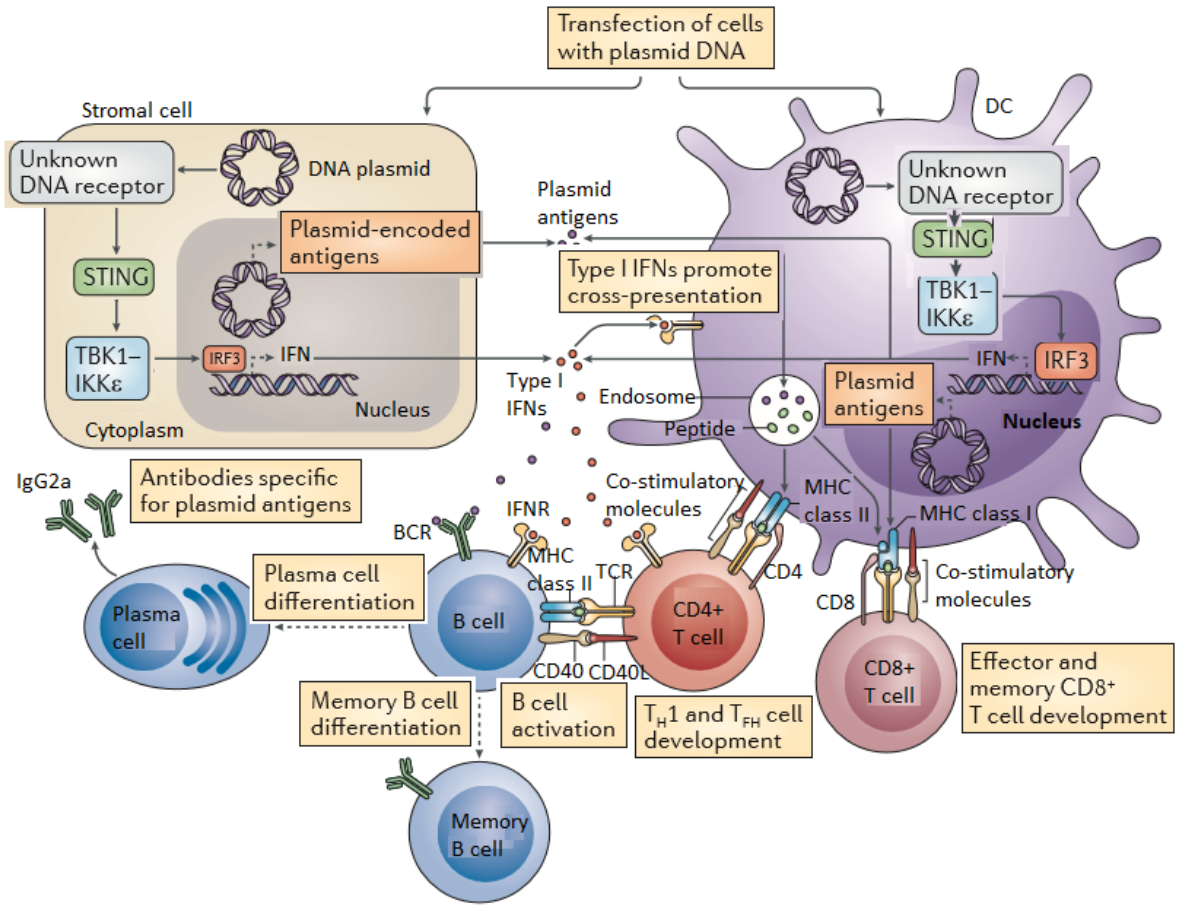

Figure 6: ***Mechanisms of DNA vaccination***. *Figure is modified from Desmet & Ishii, 2012 [33].*

One safety concern for DNA vaccines, which has slowed development of DNA vaccines, has been the risk of integration of partial or complete plasmid sequences into the host genome and induce activation of oncogenes or mutation of tumor suppressor genes or increase chromosome instability [34, 35]. Subsequent studies have showed that DNA vaccines have an extremely low probability of human genome integration [35]. For human use DNA vaccines against cancer, HIV, influenza and malaria have been explored [34, 35]. Already, several DNA vaccines have been licensed for veterinary applications; fish (infectious hematopoietic virus), dogs (melanoma), swine (growth hormone releasing hormone) and horses (West Nile virus) [35].

It has been estimated, that from 15 % to 25 % of cancers worldwide are caused by microbes including bacteria, viruses, and parasites. Bacterial and viral infections, such as *Helicobacter pylori*, HPV, hepatitis C virus, hepatitis B virus, human herpes virus, and HIV, have been established as major risk factors for many human cancers. In general, cancer vaccines are classified to preventive (or prophylactic) vaccines and treatment (or therapeutic) vaccines. Preventive vaccines are intended to prevent cancer from developing

in healthy people. It targets infectious agents that cause or contribute to the development of cancer. Therapeutic vaccines are intended to treat an existing cancer by strengthening the body's natural immune response against the cancer. Therapeutic vaccines can be divided to DC vaccines, antigen/adjuvant vaccines, whole-cell cancer vaccines, and viral vectors and DNA vaccines. DCs are stimulated with autologous cancer antigens and reinjected into the patients. This activates the immune system's T cells. While in antigen vaccines, specific protein fragments or peptides of tumor markers from antigens are used to stimulate the immune system to target tumor cells. Whole-cell vaccines are composed of attenuated or inactivated tumor cells and are usually administered with cytokines to enhance the tumor immune response. In the case of the DNA vaccines, DNA coding for a specific tumor antigen is transported into cells, which then start to make these antigen molecules [36].

3 Aims of the Study

The main aim of this study was to produce and characterize virus-like particles for coxsackievirus B1 (CVB1), and optimize CVB1-VLP production.

More specifically, the aims of the study were:

I To produce baculovirus stocks for CVB1-VLP production and produce CVB1-VLPs in insect cells.

II To set up a titration assay for CVB1-VLP baculovirus stocks using insect cells.

III To optimize CVB1-VLP production by comparing the type of insect-cell line and the flashBAC DNA variant, the Multiplicity Of Infection (MOI) used for CVB1-VLP amplification, baculovirus cultivation time, type of cell growth medium and culture volume.

IV To develop a scalable purification method for the expressed VLPs.

4 Materials and Methods

4.1 Generating baculovirus expressing CVB1-VLP

4.1.1 Design and construction of the CVB1-VLP transfer vector

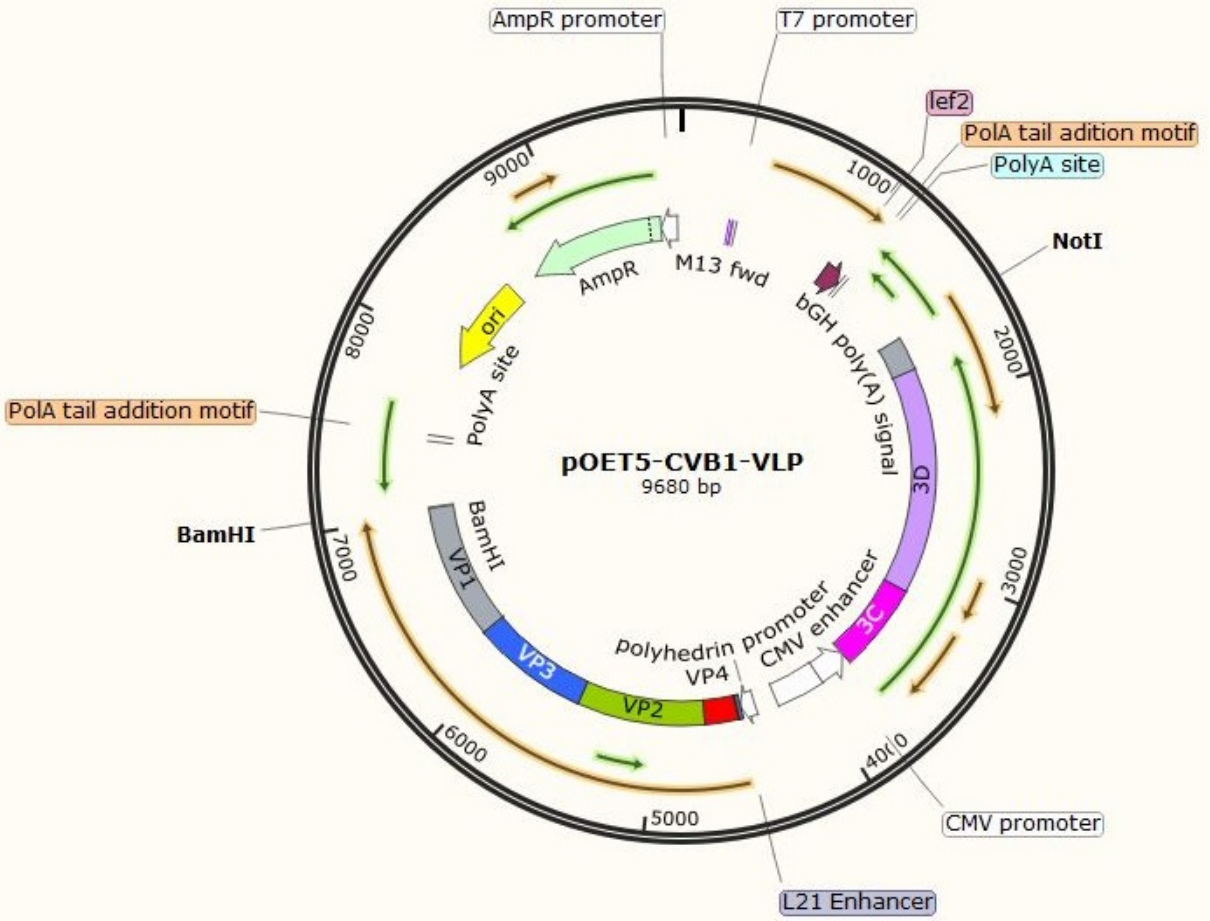

Figure 7: ***Cloning of CVB1-VLP expression cassettes into baculoviral transfer vector pOET5.***

The recombinant baculoviruses were generated according to the flashBAC user guide [7]. Baculoviral transfer vector pOET5 (Oxford Expression Technologies, Oxford, England) containing the desired inserts was ordered from GeneART Gene Synthesis service (Germany). The P1-region of the fully sequenced CVB1-strain [15] was cloned upstream of the strong Polyhedrin (Polh) promoter. 3CD protease region was cloned in a separate expression cassette upstream of the weaker CMV promoter (Figure 7).

4.1.2 Cell culture and generation of the VLP-producing recombinant baculovirus

Spodoptera frugiperda insect cells (Sf9; Invitrogen) were cultured using SFX-Insect serum-free medium (HyClone) for baculovirus production at 27^{o}C. *Trichoplusia ni* insect cells

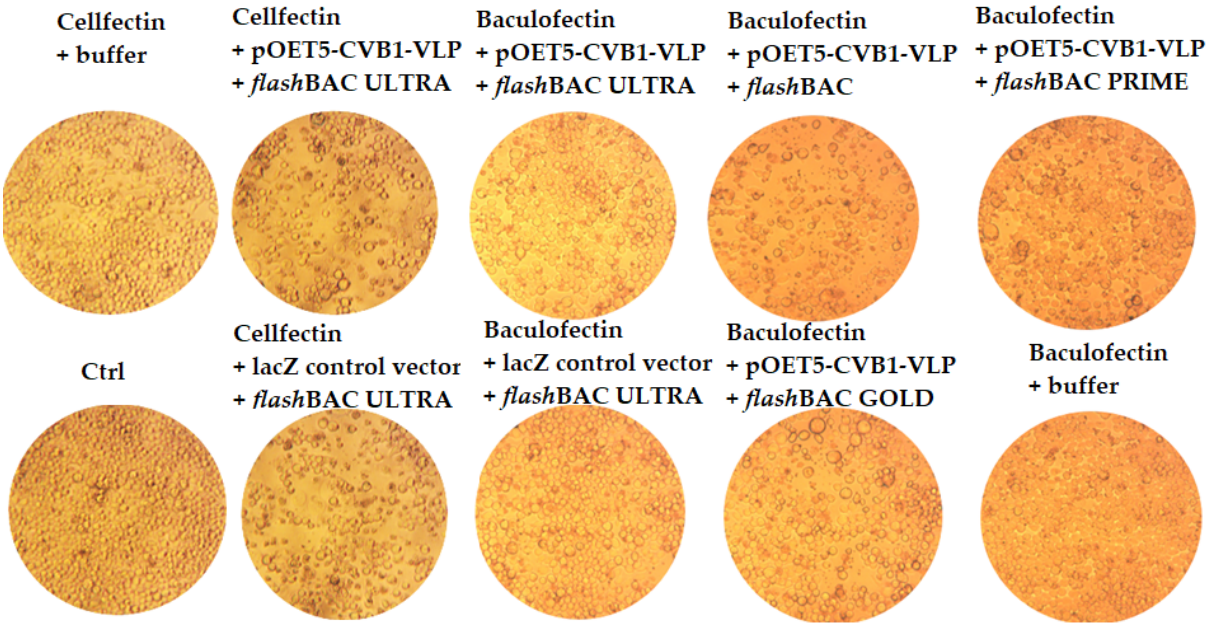

Figure 8: ***Co-transfection of Sf9 cells together with flashBAC DNA and CVB1-VLP transfer plasmid to produce the recombinant baculovirus seed stock P0.*** *P0 was harvested after 5 day incubation. LacZ-control transfer vector was used to see that co-transfection proceeded and two different transfection reagents (Cellfectin and Baculofectin II) were tested to find out the best transfection efficiency. (See Appendix Figure 24.) Image: 10X magnification, 6-well plate, middle area of wells.*

(High Five, Invitrogen) were cultured in shaker flasks at 27^{o}C using Express-Five SFM serum-free medium (Gibco) with L-glutamine (Gibco) and heparin (10 U/ml, Sigma-Aldrich). Sf21 cells were also cultured using SFX-Insect serum-free medium (HyClone).

The recombinant baculovirus was produced by co-transfecting Sf9 insect cells (viability 96 %) with different flashBAC DNA variants (flashBAC ULTRA, flashBAC GOLD, flashBAC or flashBAC PRIME)(OET, UK) together with the CVB1-VLP transfer plasmid (pOET5-CVB1-VLP). Co-transfection was done using 6-well plates: in each well 1 x 10^6 cells were plated in 2 ml total volume. Cells were allowed to attach 30 minutes before adding DNA-lipid mixture onto the cells. For each transfection reaction 8 µl reagent was added to 100 µl medium and vortexed briefly to mix, and 500 ng pOET5-CVB1-VLP plasmid and 100 ng flashBAC DNA was diluted to 100 µl medium and mixed gently. Then diluted DNA was mixed into the diluted transfection reagent and incubated 30 min in room temperature (RT). After the incubation period, the DNA-lipid mixture was diluted into 1,8 ml medium. Medium was aspirated from 6-well plate and DNA-lipid mixture was added dropwise onto the cells and incubated 5 days at 27^{o}C. See Figure 8. Two different transfection reagents were tested; Cellfectin II Reagent (Invitrogen) and BaculoFECTIN II (OET, UK). The recombinant virus was harvested from the co-transfection medium

and became the seed stock (P0) for the recombinant baculovirus. Next step was amplification of recombinant baculovirus to produce high titer stocks for the virus (P1, P2 and P3). P1 virus stock was done by inoculating 250 µl P0-virus into 50 ml insect cell culture (viability 95 %) with 1 x 10^6 cells/ml density. P1 was harvested 4 days post-infection (dpi). P2 and P3 virus stocks were produced as described for P1 virus stock.

4.1.3 Infectivity titration of the recombinant virus

Different titration methods were tried out to find the infectivity titers for different baculovirus stocks. Plaque-assay and cytotoxicity assay was tested in Sf21 insect cells unsuccessfully. However, the end-point dilution method ($TCID_{50}$) in Sf9 insect cells performed very well and is described in the following:

First, 50 µl insect cell medium was added to each well of the 96-well plate. Then 10 µl of the 1:100 diluted virus was added to each well of the third column, mixed well and then 10 µl from this was moved to the next column, etc. until well 12. 10 µl from each well of the 12^{th} column was discarded. Then 5 x 10^4 Sf9 cells (in 150 µl volume) were plated in each well and incubated at 27^oC for 7 days. Subsequently, 50 µl virus medium was collected from each well of first plate and was transferred to new 96-well plate with new cells and incubated for 5 days. Then the medium was aspirated from each well and the living cells were stained with crystal violet (50 µl/well for 5 minutes). Extra color was washed under running water and the number of living cells was compared to the number of living cells in the controls. $TCID_{50}$ value of the virus was calculated statistically using Karber's formula: $TCID_{50}$.

4.2 Optimizing the production of CVB1-VLP in insect cells

CVB1-VLPs were produced in different insect-cell lines (Sf9, Sf21 and High Five) and their yields were compared using SDS-PAGE and Western Blotting. Also, the VLP yields after different baculovirus infection times (3 to 8 days infections) and culture volumes were compared (50 - 500 ml volumes). Different flashBAC DNAs and MOI-values (0.2 - 20) were used to find optimal combination to produce VLPs.

4.3 Purification of CVB1-VLPs

4.3.1 Clarification

The supernatant and the cells were harvested and separated by centrifugation (7,068 x g for 30 min at 4^oC with Sorvall Lynx4000 and BioFlex rotor). Then the cells were

suspended in PBS and lysed with three freeze/thaw cycles or sonicated with sonicator (6 x 5 s ON/ 5 s OFF for 60 s at A = 29 %, VibraCell). After the virus release, the cell debris was pelleted by centrifugation (15,000 x g for 20 min at 4^{o}C with Sorvall Lynx4000 and F21-8x50y rotor) and the supernatants were filtered through 0.45 µm and 0.2 µm filters.

4.3.2 Concentration and buffer exchange

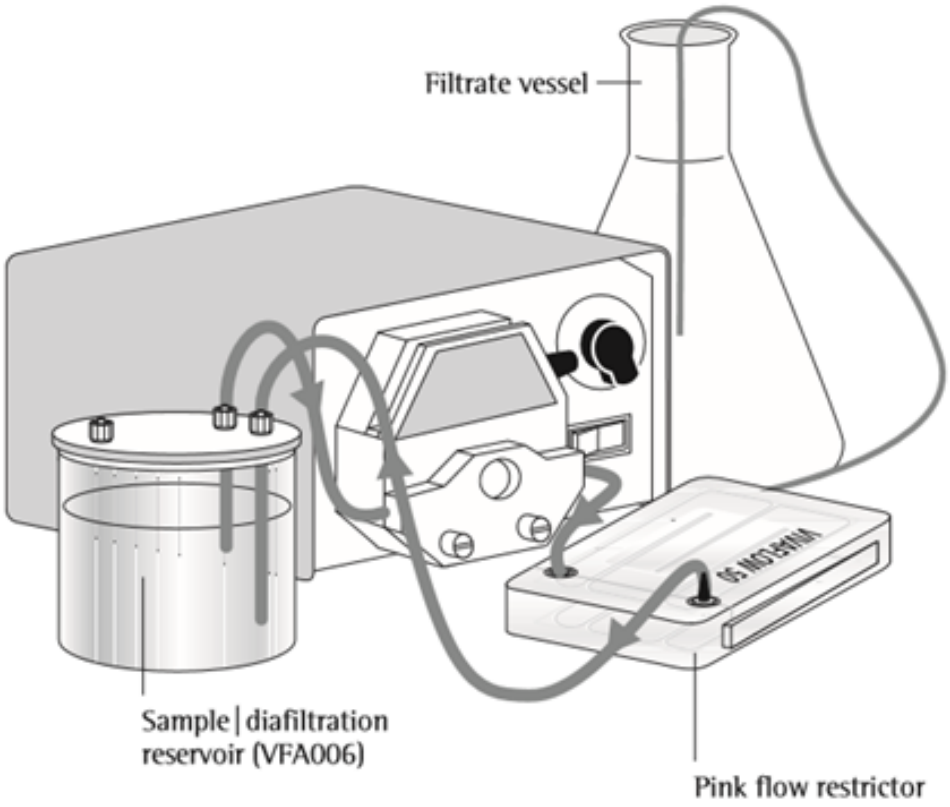

Figure 9: ***Vivaflow set up.***

The cleared supernatants of the intra- and extracellular VLP-protein extracts were concentrated by tangential flow filtration (TFF) or PEG-precipitation. Briefly, 1000 kDa MWCO membrane cassettes of the Vivaflow concentration system (Sartorius, Germany) were used in TFF (Figure 9). Some of the VLP productions were PEG-precipitated after clarification (70 g/l PEG6000 (Merck) and 22g/l NaCl was dissolved in the protein extract with magnetic stirring at 4^{o}C for 4-20 h). The buffer exchange before the ion exchange purification was done by TFF or gel filtration using HiTrap Capto 700 column (GE Healtcare) and ÄKTApurifier system (GE Healthcare). The buffer was exchanged into 20-40 mM Tris-HCl (pH 6.8-7.5), 5-10 mM $MgCl_2$, 20-40 mM NaCl with or without 0.1 % Tween80.

4.3.3 Purification and polishing

After the concentration and buffer exchange steps, the VLPs were purified by ion exchange chromatography (IEX) using High Pressure Liquid Chromatography (HPLC) (ÄKTApurifier system) or ultracentrifugation. If the VLP sample was too concentrated in salt, it was diluted with low concentration buffer (10 mM Tris-HCl pH 7.5) until the salt concentration was low enough for binding to IEX column in ÄKTApurifier-system[1]. The IEX purifications of the pretreated VLPs were performed using columns from GE Healthcare (Sweden) like HiTrap QXL (strong anion exchanger) and HiTrap DEAE FF (diethylaminoethyl, weak anion exchanger), or monolithic columns based on CIM technology from BIA Separations (Slovenia) like QA (quaternary amine, anion exchanger) and SO3 (sulfate, strong cation exchanger). Also, hydrophobic interaction chromatography (HIC) was tested with monolithic column C4 HLD (high ligand - butyl) and gel filtration chromatography with HiLoad 16/600 Superdex 75 pg (composite of cross-linked agarose and dextran). VLPs were eluted from the columns using increasing concentrations of NaCl in the respective elution buffers[2][3] with linear or stepwise gradients. Also, pH gradient was tested unsuccessfully. The VLP-containing fractions were assessed by SDS-PAGE and Western blotting using mouse anti-enterovirus clone 5-D8/1 (DAKO, Glostrup, Denmark; 1:3000) for CVB1-VLP and stored at -20^{o}C until further use.

Some VLPs were purified by ultracentrifugation with sucrose cushion pelleting. First, the VLP was PEG-precipitated and then a sucrose cushion was prepared by adding 1 ml of 50% sucrose to the bottom of 14 ml ultracentrifuge tube and 1 ml of 30% sucrose on top of that. Then VLP was added into the tube. VLP was centrifuged for 14 h at 285 000 g at 4^{o}C (XPN-100 ultracentrifuge with SW40Ti rotor). Then, the supernatant was collected in 1 ml fractions and pellet was dissolved into 500 µl PBS-0.1 % Tween80 by magnetic stirring at cold room for 20 h. Subsequently, the VLP was solubilized by resuspending up and down with syringe and insulin needle and unsoluble material was pelleted by centrifugation (13,000 x g, 5 min, +4^{o}C). VLP was collected to low protein binding tubes and stored at -80^{o}C. The collected fractions after sucrose cushion pelleting and the solubilized pellet were then analyzed with SDS-PAGE, WB and DLS.

CVB1-VLPs treated with different VLPs were purified and polished with many IEX steps. Some of purified VLPs was tried to concentrate and polish with Vivaspin (Sartorius) and PALL Macrosep (Life Science) concentrators without getting any better result.

[1]Too concentrated salt hampers most of the protein matrix interactions.
[2]Binding buffer: 20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 20 mM NaCl.
[3]Elution buffer: 20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 2 M NaCl.

4.4 Characterization of CVB1-VLPs

4.4.1 SDS-PAGE and Western Blotting

The purity of the VLP samples and the presence of the VP0, VP1 and VP3 subunits in the purified VLP batches was detected by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were denatured by heating at 100 ^{o}C for 10 min in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE in a Criterion TGX Stain-Free Precast Gel (Bio-Rad Laboratories) and visualized by UV detection with ChemDOC XRS+ (Bio-Rad Laboratories) or Oriole Fluorescent Gel Stain Diluent (Bio-Rad Laboratories) with ChemDOC XRS+.

For the detection of the expressed proteins by antibodies, the samples were first separated on SDS-PAGE gels and blotted onto a nitrocellulose membrane by Midi-run with Trans-Blot Turbo (Bio-Rad Laboratories). Then the blot was incubated in Ponceau 'S color for 10 min to detect protein ladders, which were marked with WB-marker pen (LI-COR) and Ponceau 'S color was washed away with water. Blot was blocked by incubating for 40 - 60 min in Odyssey Blocking Buffer (LI-COR). Then it was incubated in primary antibodies: for 1 h in monoclonal mouse anti-enterovirus clone 5-D8/1 (DAKO, Glostrup, Denmark) for detection of VP1 and monoclonal mouse anti-baculovirus gp64 clone AcV5 (Santa Cruz Biotechnology Inc., Heidelberg, Germany) for detection of baculoviral contamination at a dilution 1:3000 and 1:2000, respectively. 3 x 7 min TBST washes were performed between incubations. Then the blot was incubated in goat anti-mouse IRDYE secondary antibody using dilution 1:5000 for 1.5 – 2 h. After secondary incubation, the blot was washed 3 x 7 minutes with TBST and finally with TBS to remove the tween that might interfere with the imaging of the blot. Blots were read using a LI-COR Odyssey CLX.

4.4.2 Total protein concentrations

The total protein concentrations of the fractions were analyzed using the Pierce BCA Protein Assay kit (Thermo Scientific) and the absorbance was measured with EnVision Multilabel Plate Reader (PerkinElmer) or NanoDrop2000 UV-Vis Spectrophotometer (Thermo Scientific). The VLP purity was estimated by densitometry analysis with ImageLab software (Bio-Rad) from the gel.

4.4.3 Dynamic light scattering

A dynamic light scattering (DLS) instrument Zetasizer Nano ZS (Malvern Instrument Ltd., UK) equipped with a HeNe gas laser (λ = 633 nm) was used to determine the

hydrodynamic diameter of the VLP preparations. The samples were measured at room temperature with predefined Standard Operating Procedures (SOPs). The hydrodynamic diameter was determined from a cumulant analysis of six consecutive measurements, each containing 15 readings over 10-second intervals and plotted as the mean hydrodynamic diameter of particle population with accompanying standard error.

4.4.4 Transmission electron microscopy

The VLP morphology was observed by transmission electron microscopy (TEM). This step was performed by Docent Varpu Marjomäki (University of Jyväskylä, Finland). For the TEM analysis, a small aliquot sample was briefly bound to copper grids, which were air-dried before visualization with a JEM-1400 (JEOL, Tokyo, Japan).

5 Results

5.1 Production of VLPs

5.1.1 Optimization of CVB1-VLP production in insect cells

The VLP-producing recombinant baculovirus stocks were successfully produced in Sf9-cells as demostrated by Western blotting of baculovirus surface protein gp64, stained with mAb gp64 clone AcV5. CVB1-VLP was also produced while the baculovirus stocks were amplified, demonstrated by Western blotting of CVB1 capsid protein VP1 (31 kDa) stained with mAb 5-D8/1 (Figure 10).

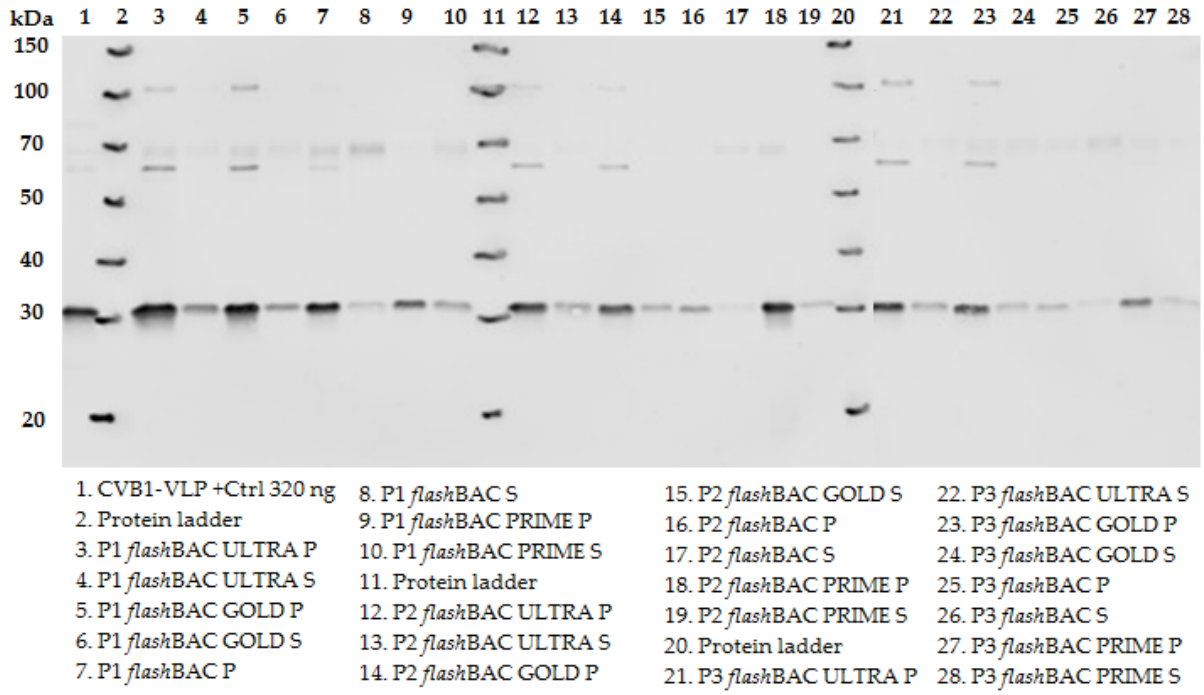

Figure 10: ***Characterization of different CVB1-VLP recombinant baculovirus stocks by Western blotting.*** *Baculovirus surface protein gp64 (64 kDa) could be found in the intracellular fraction (cell pellets, P) and in the extracellular fraction (supernatant, S).*

The infectivity titers for CVB1-VLP producing baculoviruses (Table 1) were successfully determined by end-point dilution assay.

First, we tried to set up the plaque assay for the baculoviruses, but clear enough plaques were not forming in Sf21 or Sf9 cells. The technical support in OET instructed us to use Sf21 cells with 10 % FBS, because culturing the cells without FBS or with low quality FBS might lead into inadequate closure of the cell monolayer and therefore plaques cannot form. However, we did not succeed in using FBS with insect cells, because the culture medium designed to be used with FBS required CO_2 buffering system and we did not have instrument available.

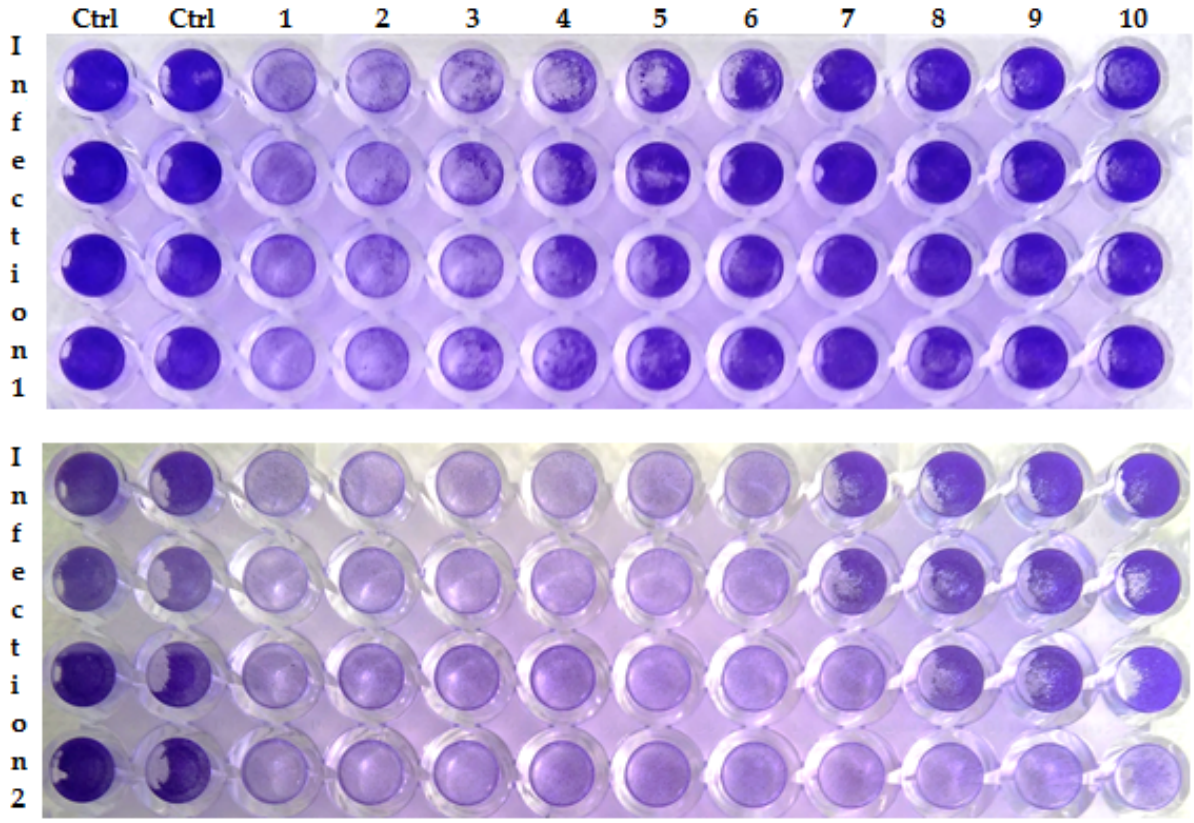

Figure 11: ***Infectivity titration of baculovirus in Sf9-cells stocks by end-point dilution method.*** *The infectivity titer was calculated after the second infection period, when the cytopathic effect was more clear.*

The end-point dilution assay that was utilized based on the method of Reed and Muench (1938). The method was adapted so, that after one week baculovirus infection period, different baculovirus dilutions were transferred to another 96-well plate with fresh cells. This protocol produced clear CPE and virus infectivity titer could be determined reliably (Figure 11).

Table 1: ***The infectivity titers of different CVB1-VLP baculovirus stocks.*** *The $TCID_{50}$ titer per ml was converted to PFU/ml by multiplying the $TCID_{50}$ titer by 0.7.*

CVB1-VLP	P2	P3
flashBAC ULTRA	2.5×10^8	3.6×10^9
flashBAC GOLD	3.9×10^8	2.5×10^8
flashBAC	3.9×10^8	6.4×10^7
flashBAC PRIME	2.6×10^7	6.9×10^6

Subsequently, CVB1-VLP production efficiency was compared in three different insect cell lines and cell growth media. The analysis was performed by VP1 protein quantification by Western blotting. The best CVB1-VLP production efficiency was in High Five cells, whereas Gibco ExpressFive and Lonza insect-XPRESS media had equally good production efficiences (Figure 12).

The production efficiencies of different flashBAC DNA variants were compared in High Five cells and the best production efficiencies were in flashBAC ULTRA and flashBAC GOLD and there was no significant difference between these two above mentioned variants (the analysis was performed by VP1 protein quantification by Western blotting). (Figure 13).

The production efficiencies of CVB1-VLP with different MOI values were compared in High Five cells. MOI values 0.2, 1, 5, 10 and 20 were tested and MOI 0.2 and 1 seemed to give the highest production yields (the analysis was performed by VP1 protein quantification by Western blot). (Figure 14).

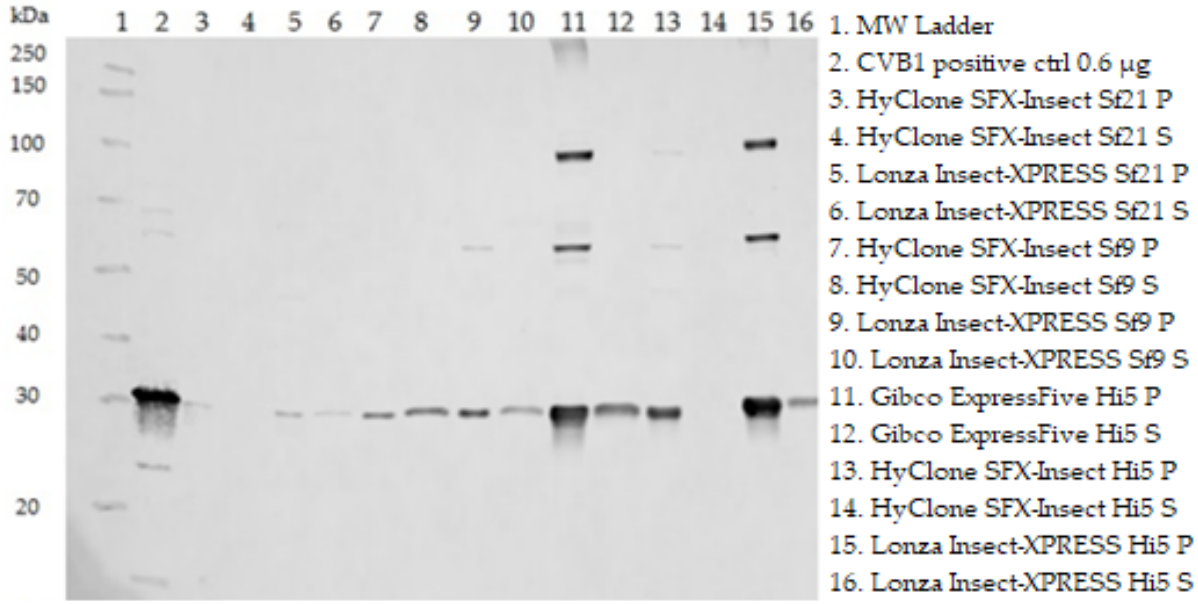

Figure 12: ***Effect of the insect cell line and cell growth medium on the CVB1-VLP production efficiency.*** *All the productions were performed using CVB1-VLP P2 flashBAC ULTRA baculovirus, 5 days infection period, 50 ml culture volume and MOI 5.*

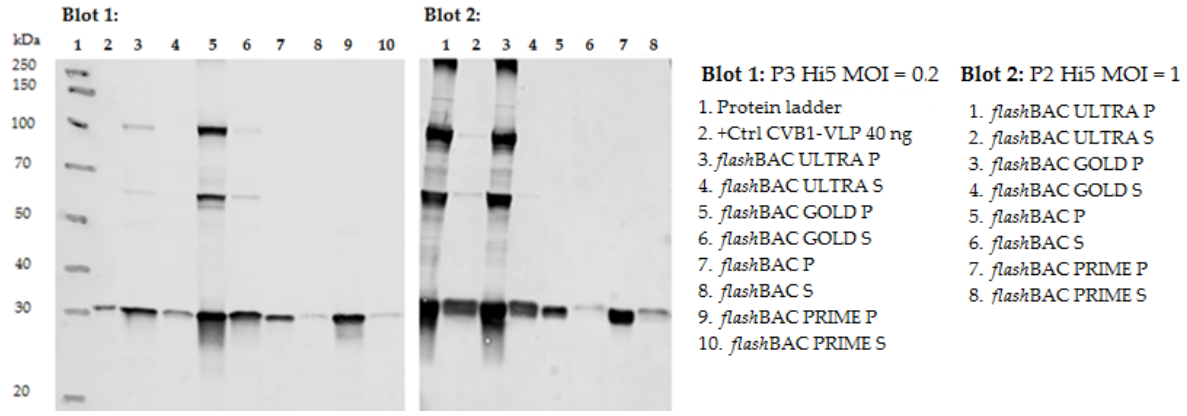

Figure 13: ***Effect of the different flashBAC DNA variants on the CVB1-VLP production efficiency.*** *The CVB1-VLP productions were performed in High Five cells using either P2- or P3 baculovirus stock with 50 ml culture volume and MOI 0.2 or 1.*

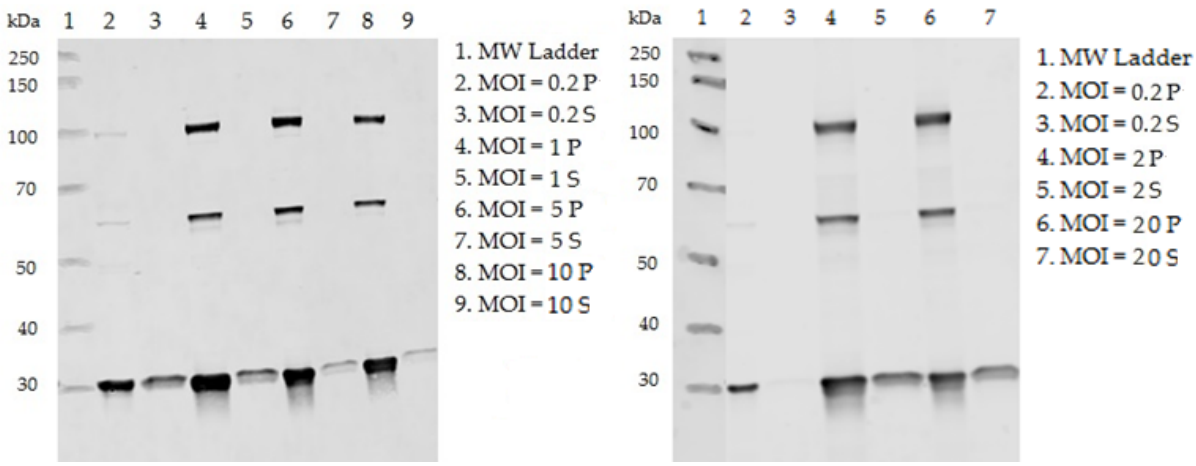

Figure 14: ***Effect of the MOI value in the CVB1-VLP production efficiency.*** *The CVB1-VLP productions were performed in High Five cells using P2 flashBAC ULTRA virus stock and 100 ml culture volume with 5 days infection period.*

Finally, different baculovirus cultivation times and culture volumes were compared for finding out the most optimal production conditions for CVB1-VLPs (the analysis was performed by VP1 protein quantification by Western blot). The production efficiency decreased when the culture volume was increased, the best culture efficiency being in the 50 ml culture. There was no significant difference between four to six day incubation periods, but three day incubation produced significantly lower protein yield compared to six day cultivation time (Figure 15).

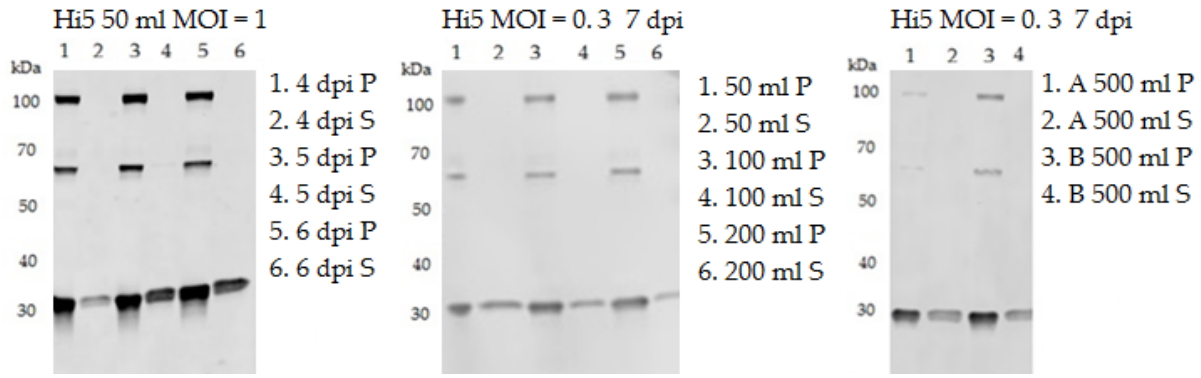

Figure 15: ***Effect of the baculovirus cultivation time and culture volume on the CVB1-VLP production efficiency*** *(CVB1-VLP P2 flashBAC ULTRA virus stock and High Five cells).*

5.1.2 CVB1-VLP purifications

Various different purification methods were tested for CVB1-VLP and the most relevant ones are shown in the following.

Purification of CVB1-VLP by sucrose cushion pelleting

First, we wanted to see how the extra- and intracellular CVB1-VLP yields differ. The first purification batch was therefore divided in batches #1a and #1b. #1a contained the extracellular VLP protein and #1b contained the intracellular VLP protein dissolved in PBS-0.1 % Tween80. The intracellular protein was released from the cells with three freeze-thaw cycles and the cleared supernatants from #1a and #1b were PEG-precipitated and pelleted through 30/50% sucrose cushion with ultracentrifugation. After the ultracentrifugation, the supernatant was collected in 1 ml fractions and pellet was dissolved into 500 µl PBS-0.1 % Tween80. Fractions F13, F12 and solubilized pellet contained the extracellular VLP, whereas fractions F13, F11 and solubilized pellet contained the intracellular VLP. However, the purity was compromised in both #1a and #1b. Both batches contained baculovirus and CVB1-VLP concentration in #1b was very low. (See Figure 16 and Table 2).

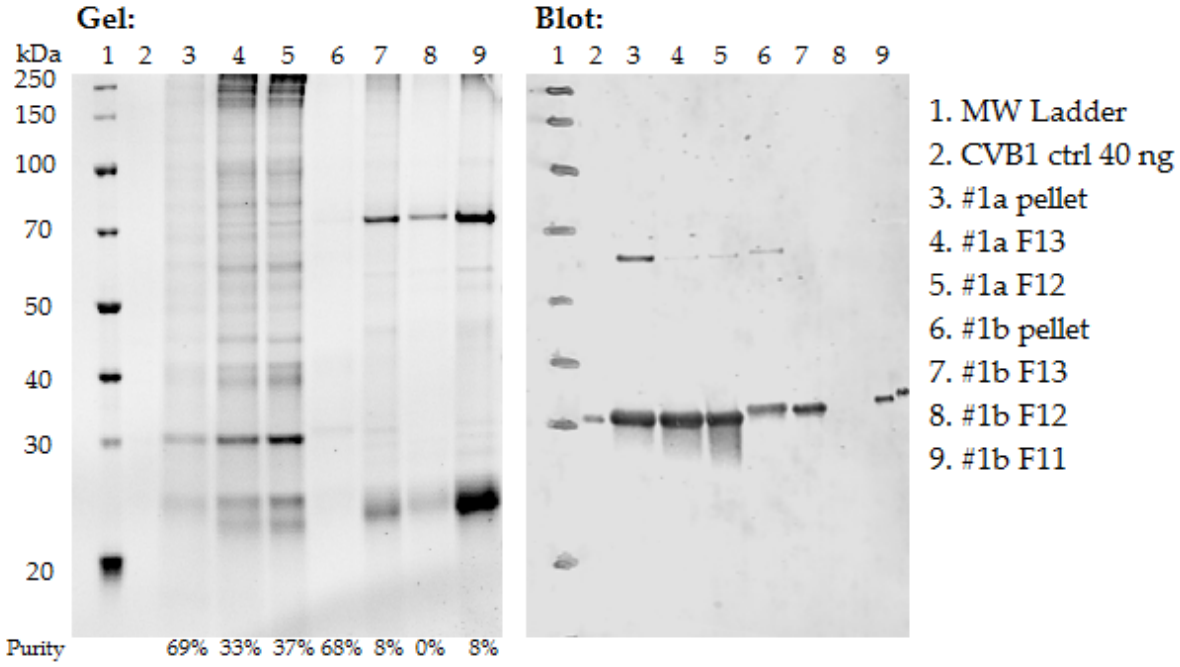

Figure 16: ***Characterization of the purified CVB1-VLP #1a on SDS-PAGE gel and Western Blot.*** *Baculovirus surface protein gp64 was stained with mAb gp64 clone AcV5 and CVB1 capsid protein VP1 was stained with mAb 5-D8/1.*

Table 2: ***Purity and yield of CVB1-VLP #1.*** *The yield was approximately 5.25 mg VLPs per liter of insect cell culture, when 50 ml cultures were pooled together.*

Batch	Purity %	CVB1-VLP (mg/ml)	CVB1-VLP Yield (mg)
#1a pellet	69	0.76	0.31
#1a F13	33	0.35	0.17
#1a F12	37	0.35	0.35
#1b pellet	68	0.63	0.10
#1b F13	8	0.06	0.03
#1b F11	8	0.09	0.09

Purification of CVB1-VLP by ion exchange chromatography

We wanted to develop a purification method, that would be scalable for several liters of production medium. We also wanted to develop a method producing the highest possible yield and purity for CVB1-VLP. We have previously seen, that concentrating CVB1 virus with PEG-precipitation results in low virus recovery, the loss of virus being approximately 85% during PEG-precipitation step [37]. Therefore, we utilized tangential flow filtration and 1000 kDa MWCO cassette for the first concentration step. However, when two identical CVB1-VLP production batches were concentrated using either PEG-precipitation or

tangential flow filtration, the recovery or the purity did not differ significantly between these methods (data not shown).

#5a CVB1-VLPs were concentrated by TFF using a 1000 kDa MWCO membrane and after that ran through HiTrap Capto core 700 medium and were collected in the flow through (FT). FT was diluted with 10 mM Tris buffer (pH 7.5) until the conductivity was 2.5 mS and was then purified further using SO3 column at a NaCl concentration between 20 - 100 mM (Figure 17).

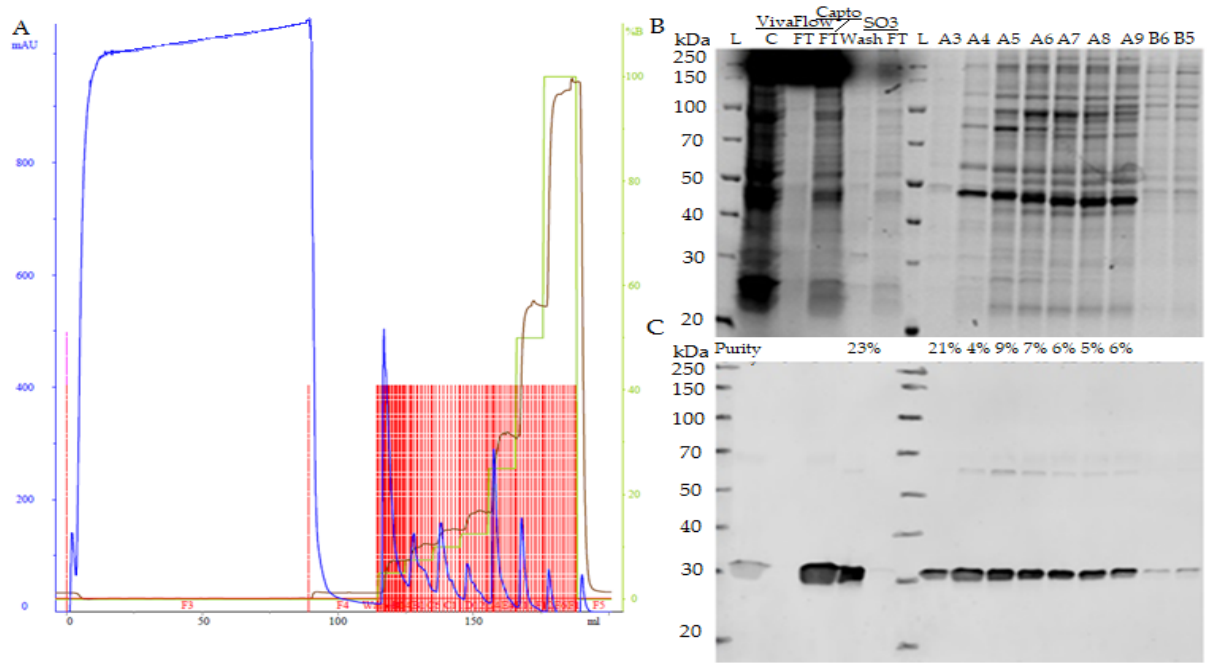

Figure 17: ***Characterization of the chromatography purified CVB1-VLP #5a.*** *A) TFF-concentrated VLPs were loaded onto a cation exchange column and were eluted from the column with a stepwise gradient using 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 M NaCl, 0.1% Tween80 as the elution buffer. A flow rate of 1 ml/min was used. B) Analysis of the SDS-PAGE gel stained with stainfree dye technology showed that VLP eluted at a NaCl concentration 20-100 mM. C) Western blot analysis of the baculovirus surface protein gp64 and VP1 capsid protein confirmed the presence of these proteins in the purified fractions at sizes 64 and 31 kDa respectively.*

The VLP containing fractions from #5a intermediate purification were pooled and further subjected in purification with SO3-column (hereinafter referred to as #5a2). The VLPs could be captured to the column and they eluted from the column in stepwise gradient at NaCl concentration 200 mM (Figure 18). The purity of the A10 peak fraction was 54% and the yield was only 200 µg / liter of Sf9 production medium.

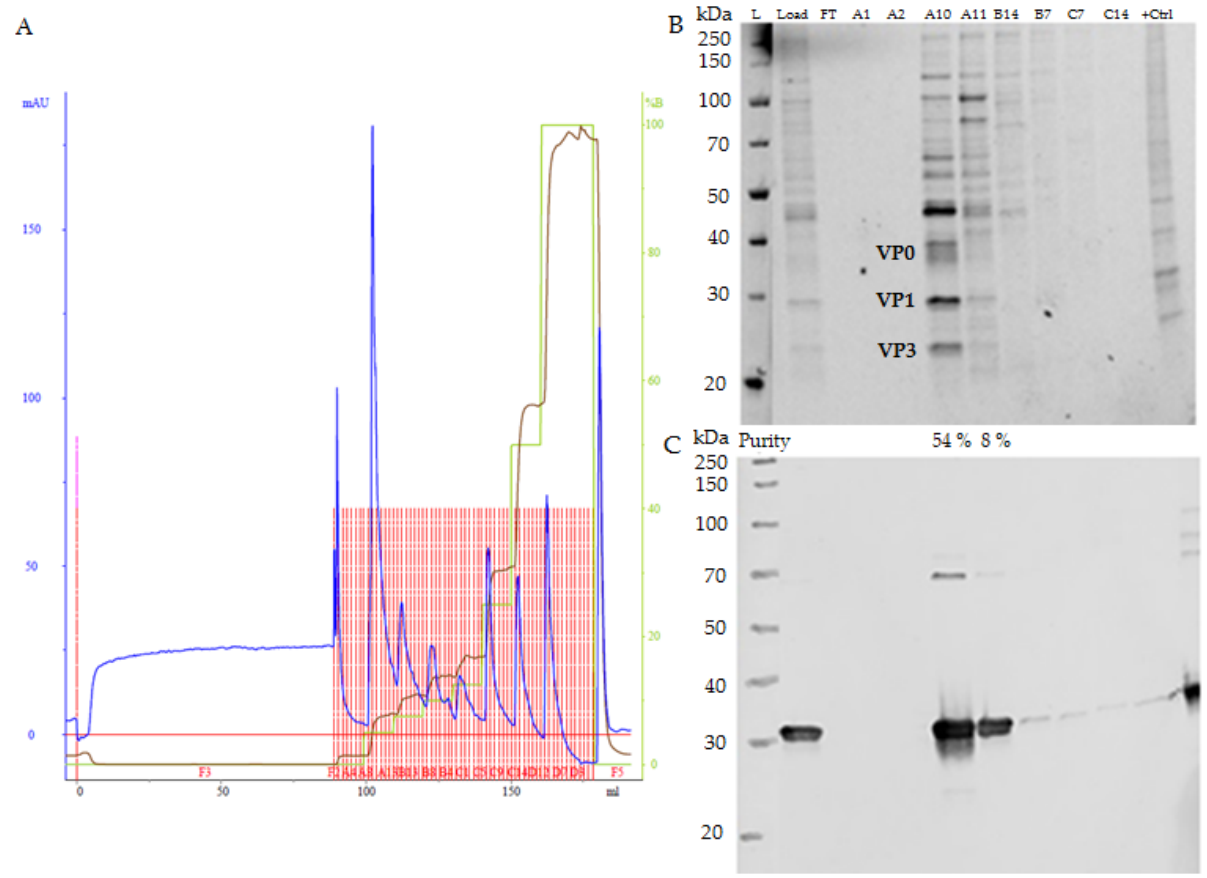

Figure 18: ***Characterization of the chromatography purified CVB1-VLP #5a2.*** *A) VLP-containing fractions were loaded onto a cation exchange column and were eluted from the column with a stepwise gradient using 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 M NaCl, 0.1% Tween80 as the elution buffer. A flow rate of 1 ml/min was used. B) Analysis of the SDS-PAGE gel stained with stainfree dye technology showed that VLP was efficiently concentrated during the purification process in fraction A10, but the purity was only 54%. C) Western blot analysis of the baculovirus surface protein gp64 and VP1 capsid protein confirmed the presence of these proteins in the purified fractions at sizes 64 and 31 kDa respectively.*

#8a VLPs were concentrated by TFF with a 1000 kDa MWCO membrane and the buffer was exchanged to binding buffer (40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 40 mM NaCl, 0.1% Tween80) using the same membrane. VLP was then purified using strong anion exchange column (CIMmultus QA-1). The VLPs were in the FT and wash fractions, whereas large proportion of the impurities bound to the column (Figure 19).

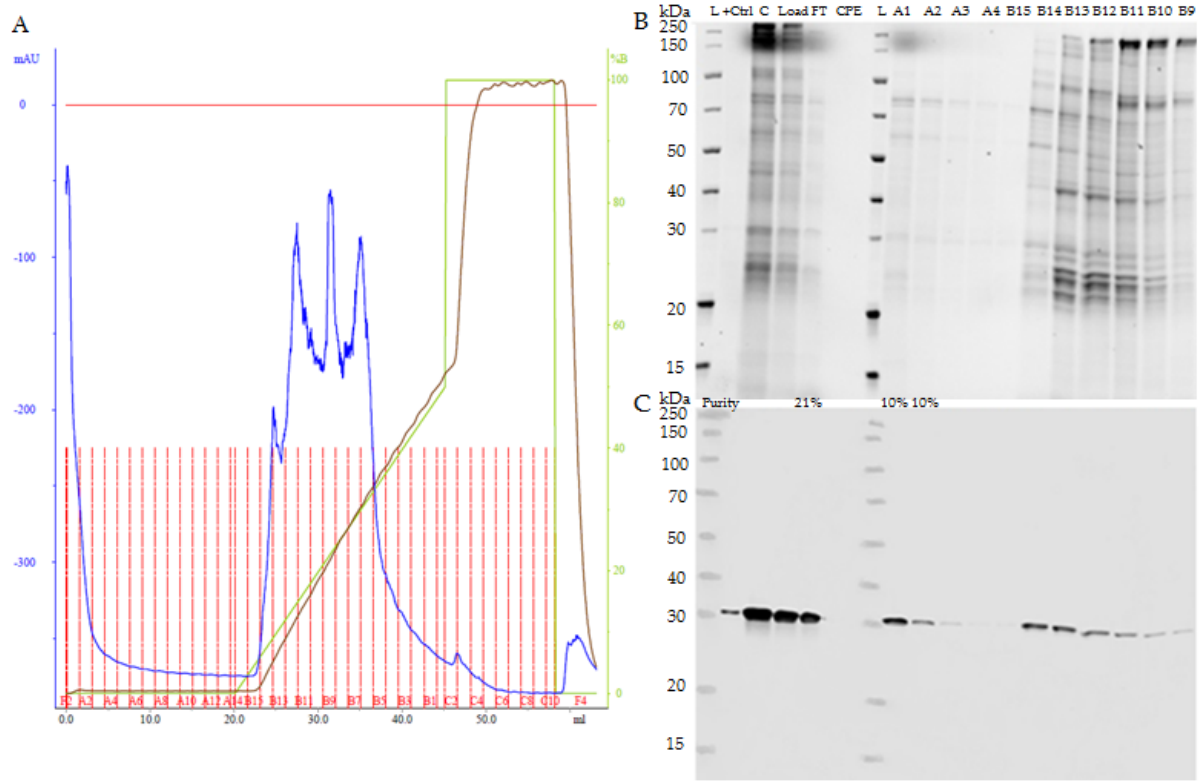

Figure 19: ***Characterization of the chromatography purified CVB1-VLP #8a.*** *A) TFF-concentrated VLPs were loaded onto a anion exchange column and were eluted from the column with stepwise and linear gradients using 40 mM Tris-HCl (pH 7.5), 10 mM MgCl, 2 M NaCl, 0.1% Tween80 as the elution buffer. A flow rate of 1 ml/min was used. B) Analysis of the SDS-PAGE gel stained with stainfree dye technology showed that most of the impurities bound to the column. C) Western blot analysis of the baculovirus surface protein gp64 and VP1 capsid protein confirmed the presence of these proteins in the purified fractions at sizes 64 and 31 kDa respectively.*

The VLP containing FT and wash fractions from #8a were pooled and further purified using SO3 strong cation exchange column (CIMmultus SO3-1) using the same binding buffer as in the previous step (hereinafter refereed to as #8a2). Again, the VLPs were in the FT and wash fractions, whereas impurities bound to the column (Figure 20).

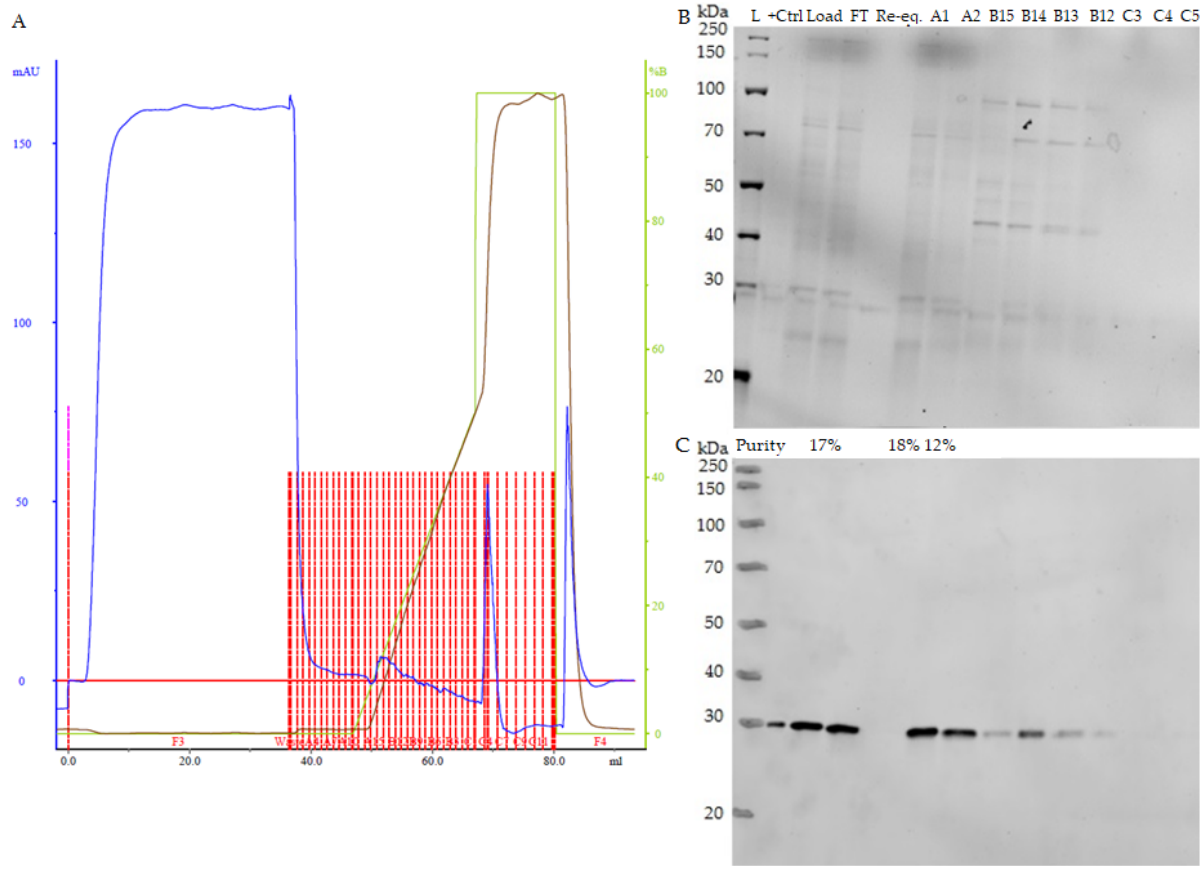

Figure 20: ***Characterization of the chromatography purified CVB1-VLP #8a2.*** *A) VLP-containing fractions were loaded onto a cation exchange column and were eluted from the column with a stepwise gradient using 40 mM Tris pH 7.5, 10 mM $MgCl_2$, 2 M NaCl, 0.1% Tween80 as the elution buffer. A flow rate of 1 ml/min was used. B) Analysis of the SDS-PAGE gel stained with stainfree dye technology showed that the impurities bound to the column. C) Western blot analysis of the baculovirus surface protein gp64 and VP1 capsid protein confirmed the presence of these proteins in the purified fractions at sizes 64 and 31 kDa respectively.*

FT and wash fractions containing the VLPs (from #8a2) were pooled and loaded on the CIMmultus SO3-1 column using binding buffer with lowered ionic strength (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 20 mM NaCl, 0.1% Tween80). The VLP was captured to the column and it eluted out of the column in one peak and two side fractions at 180 mM NaCl concentration. Analysis of purified VLP by SDS-PAGE showed fractions B12, B11 and B10 had a high purity (∼100%, ∼99% and ∼98% respectively) and the presence of proteins approximately 37, 31 and 26 kDa size (Figure 21), which correspond to the molecular weights of the capsid proteins VP0, VP1 and VP3. The yield for highly pure CVB1-VLP was 0.6 mg per liter of High Five cell culture.

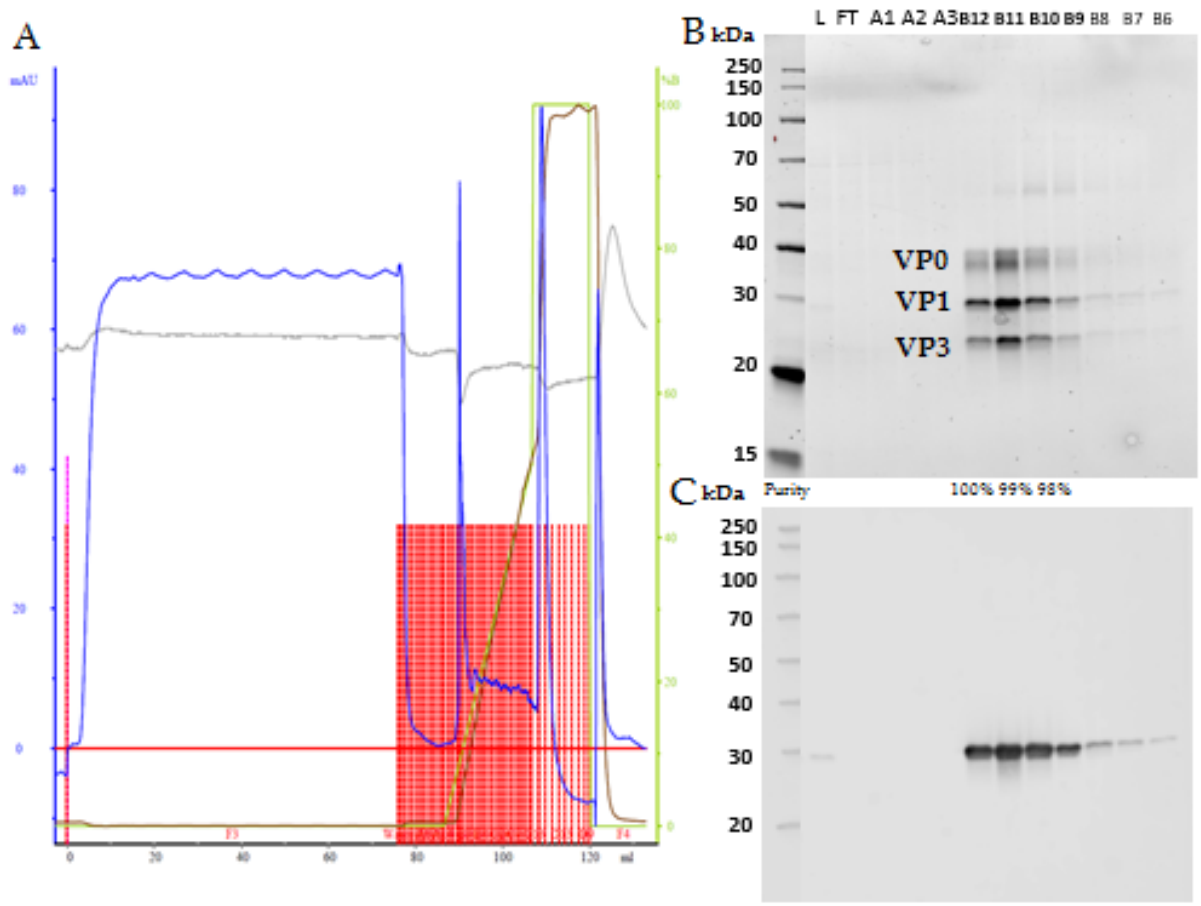

Figure 21: ***Characterization of the chromatography purified CVB1-VLP #8a3.*** *A) VLP-containing fractions from #8a2 were loaded onto a cation exchange column and were eluted from the column with a stepwise gradient using 20 mM Tris pH 7.5, 5 mM $MgCl_2$, 2 M NaCl, 0.1% Tween80 as the elution buffer. A flow rate of 1 ml/min was used. B) Analysis of the SDS-PAGE gel stained with stainfree dye technology showed that VLP was efficiently concentrated during the purification process in fractions B12-B10 with high purity. C) Western blot analysis of the baculovirus surface protein gp64 and VP1 capsid protein confirmed the presence of these proteins in the purified fractions at sizes 64 and 31 kDa respectively.*

5.2 Biophysical characterization of VLPs

5.2.1 The purified VLPs show correct size and morphology

Dynamic light scattering (DLS) was used to determine the size of the purified VLPs in the different VLP preparations. The particle size analysis of the purified VLPs revealed the presence of particles with hydrodynamic diameter between 21 and 42 nm. CVB1-VLP particle size and homogeneity was studied after each purification in the buffers that were used in the final step of the purification. The particle diameter sizes were relatively same for batches #1a and #5a2 (∼29 nm and ∼29 nm respetively). For batch #1b it was ∼42 nm and for batch #8a3 it was ∼ 25 nm. According to polydispersity indexes (PdI), batches #1b and #8a3 were quite monodisperse (PdI: 0.22 and 0.13 respectively),

whereas batches #1a and #5a2 were more polydisperse (PdI: 0.52 and 0.51 respectively). See Figure 22.

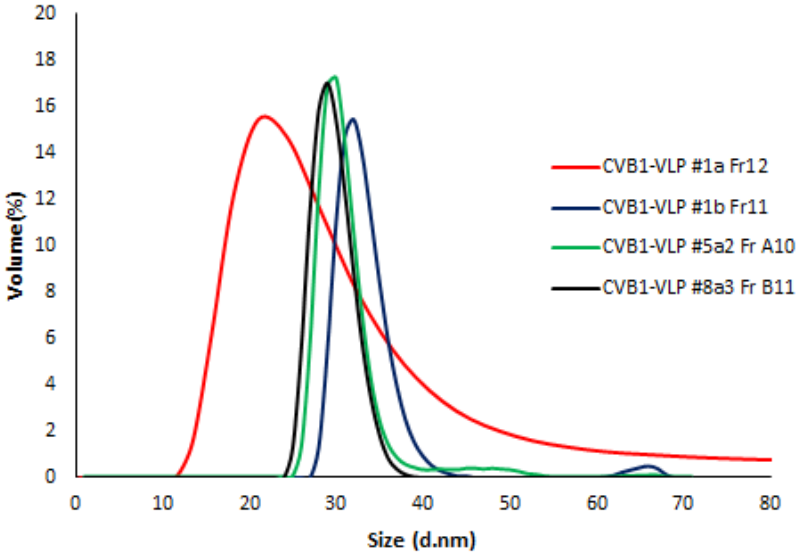

Figure 22: ***The average sizes and volume distributions of the VLPs analyzed by DLS.*** *Purified VLP #1a contained 97% particles (determined by particle volume) with a hydrodynamic diameter of 29 nm. The volume distributions and average sizes of purified VLPs #1b Fr11, #5a2 Fr A10 and #8a3 Fr B11 were 100/97/100 % and 42/29/25 nm respectively.*

The transmission Electron Micrograph analysis (TEM) revealed icosahedral VLP particles for the chromatography purified VLP-batches #5a2 (Figure 23 A) and #8a3 (Figure 23 B).

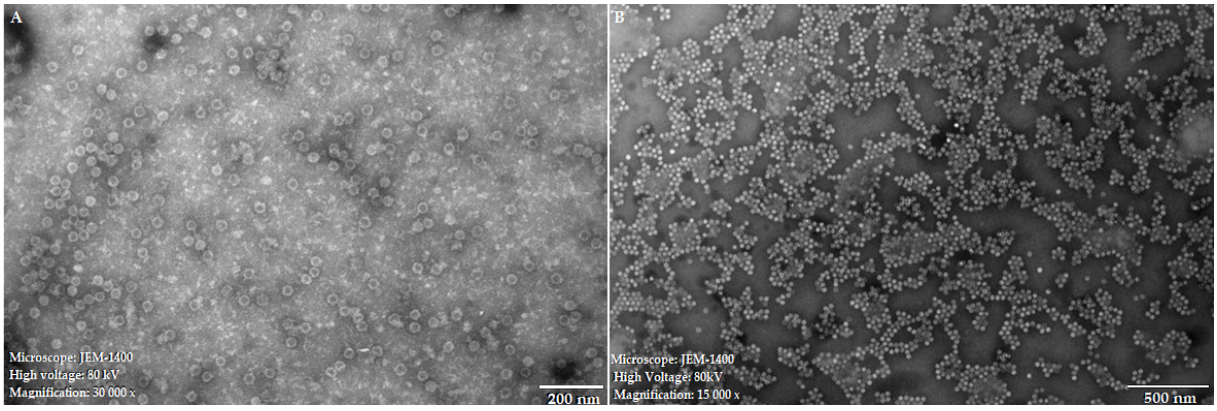

Figure 23: ***Transmission Electron Micrographs of chromatography purified CVB1-VLPs.*** *A) #5a2 (purity 54 %) and B) #8a3 B12 (purity 100 %). Scale bars 200 nm and 500 nm.*

6 Discussion

We aimed to produce, purify and characterize CVB1-VLP in this study. During the course of this study, various steps in the CVB1-VLP production were optimized including the type of insect cell line and flashBAC DNA variant, the Multiplicity Of Infection (MOI) used for CVB1-VLP amplification, baculovirus cultivation time, type of cell growth medium and culture volumes and the CVB1-VLP purification method. VLPs were concentrated either using tangential flow filtration or PEG-precipitation and purified using multi-step ion exchange chromatography (IEX).

6.1 Evaluation of results

Previously, several studies have shown various successfully produced VLPs in different insect cell lines [6, 8]. Usually VLPs have been produced in Sf9-cells, but there are also studies where High Five insect cell line have been used in VLP production. Compared to Sf9-cells, High Five cells have five to ten fold higher secreted expression levels for some VLP-proteins. High Five cells double much faster than Sf9 and Sf21, which might explain the higher secretion levels for some VLPs [38]. 3-6 days baculovirus infection periods have been used in other studies, but the best VLP production efficiencies have been obtained using 4-5 days baculovirus infection periods [6, 8].

CVB3-VLP produced previously in our laboratory, was generated using Bac-to-Bac Baculovirus Expression System (Invitrogen). However, the yields for pure CVB3-VLP have been previously 0.5 mg VLPs per liter of insect cell culture [6]. Therefore, we wanted to produce enhanced expression levels for the CVB1-VLPs and experimented the flashBAC Baculovirus Expression System for CVB1-VLP. The flashBAC system has been recently shown to enhance the expression level for another VLP of the enterovirus family (namely EV71-VLP) significantly [8]. In this study, we wanted to find out if the flashBAC system in conjunction with better design of the expression cassettes could give enhanced VLP yields. Here, the CVB1-VLP expressing P1 polyprotein was placed upstream of strong polyhedrin promoter and 3CD protease upstream of the weaker CMV promoter.

VLPs produced with the baculovirus insect cell expression system are harvested from the infected cell culture medium, containing the VLPs, baculoviruses and residual proteins from the insect cells. Therefore before the VLPs can be used e.g. as vaccines, they usually need to be purified with multi-step purification processes. However, during each purification step, some VLP protein is lost affecting to the final VLP yield. Generally in the field, both ultracentrifugation and IEX are used for purifying VLPs [6, 8].

6.1.1 Optimizing VLP productions

The VLP-producing recombinant baculovirus stocks were produced successfully and the infectivity titers were between 6.9×10^6 and 3.6×10^9 PFU/ml. According to the Bac-to-Bac instruction manual, baculovirus titers that are higher than 10^7 should produce VLPs [39]. Almost all virus stocks exceeded this value except the baculovirus titer of flashBAC P3. The most productive virus stocks were in P2 flashBAC ULTRA (it expressed 1.2 times more extracellular VLP than P2 GOLD) in High Five culture. Previously, flashBAC GOLD has been shown to give high yields of VLPs for EV71 [8]. According to the flashBAC user guide, flashBAC GOLD is ideal for protein secretion. PRIME is a wild type genome, but superior for producing some VLPs [7]. Here, it was shown that the production level of flashBAC PRIME for CVB1-VLPs was very low. FlashBAC GOLD and ULTRA variants have some gene deletions. These deletions prevent production of non-essential proteins like chitin and more of the specific VLP protein should be produced.

High Five insect cell line turned out to be the most efficient insect cell line for CVB1-VLP production. Interestingly, some unprocessed VLPs of approximately 94 kDa size could be detected by Western Blotting of the EV VLP1-region specific antibody (Figures 12-15 cell pellets). This indicates that the VLP is expressed in High Five cells so efficiently that everything cannot be processed into mature VLP. In the current study, the 3CD protease encoding region of enterovirus was placed upstream of CMV promoter, which is a weak promoter in insect cells. Therefore, it might be possible to enhance the processing of the P1-polyprotein into mature VLP capsid subunits by placing the 3CD protease upstream of stronger promoter such as p10. However, initially we wanted to use weak promoter in front of 3CD-protease, because high concentration of protease during VLP production might decrease cell stability and increase the burden of virus-infected cells, leading in low target protein yields. Also, it might be possible to increase the level of processed VLP by lowering the culturing temperature from 27 degrees to 23 degrees, as it should slow the doubling time of the cells, but this was not tested during this study.

The multiplicity of infection (MOI) values from 0.2 to 20 were compared and MOI 1 turned out to be most productive. According to quantization of the VP1 protein from the Western blot, it produced at least 1.5 x more VLPs than MOI 0.2, 5 or 10. Previously, for EV71-VLP MOI 10 has showed to be most productive [8].

Culture volumes from 50 ml to 500 ml were used and compared. We were not able to scale up the production volumes efficiently. The highest yield was with 50 ml culture (when High Five cells were used). The yield was 3 x higher using 50 ml culture than with 500 ml culture.

6.1.2 CVB1-VLP purification

We have shown in our previous study, that CVB1-virus recovery after the PEG-precipitation step is only 15% [37]. According to WB quantification of the VP1 proteins from different stages of the CVB1-VLP purification in this study, the recovery of the VLPs was from 20 to 60%.

100% purity for the CVB1-VLP was achieved using IEX, whereas maximum 69% purity was obtained using ultracentrifugation. When testing different columns and buffers for the IEX purification, we noticed that most of the impurities bind to QA column, whereas VLP does not bind to the column. Therefore, this column was used for the first intermediate purification to remove most of the impurities. Then the SO3 column with high salt concentration was used to remove most of the remaining impurities. Third ion exchange chromatography step using SO3 column successfully captured and concentrated the VLPs. Previously, QA or SO3 columns have been used in the purification of CVB3-VLPs [6].

With ultracentrifugation the total yield was 5.25 mg/l (4.15 mg/l extracellular VLP and 1.1 mg/l intracellular VLP), when several 50 ml productions were pooled, but the purity was compromised after the ultracentrifugation. The total VLP yield after IEX purifications was at the same level as with the CVB3-VLP, being 0.6 mg per liter production medium.

7 Conclusions

The main goal of this thesis was to produce and characterize virus-like particles (VLPs) for coxsackievirus B1 (CVB1). The production level was optimized by comparing different factors. High Five insect cell line turned out to be most productive cell line for CVB1-VLP. The best production efficiencies were reached with flashBAC GOLD or ULTRA. Five days baculovirus infection period was found to be most efficient in terms of VLP yield. When the culture volume was scaled up, the yield dropped significantly, the 50-ml cell culture being the most productive. MOI 1 seemed to have the best production yield. An efficient vaccine production protocol for CVB1-VLP was found. The clarification step consisted of centrifugation and filtration through 0.45 µm and 0.2 µm filters. Concentration and buffer exchange was done with tangential flow filtration using Vivaflow system (MWCO 1000 kDa filter). Intermediate purification (removal of impurities) was done with QA and SO3 columns with high salt concentrations (90 mM) and capture step with SO3 with half lower salt concentration (45 mM). The same yield was achieved for CVB1-VLP as for CVB3-VLP previosly (0.6 mg ∼100% pure VLP per liter of production medium).

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9 Appendixes

9.1 LacZ control vector

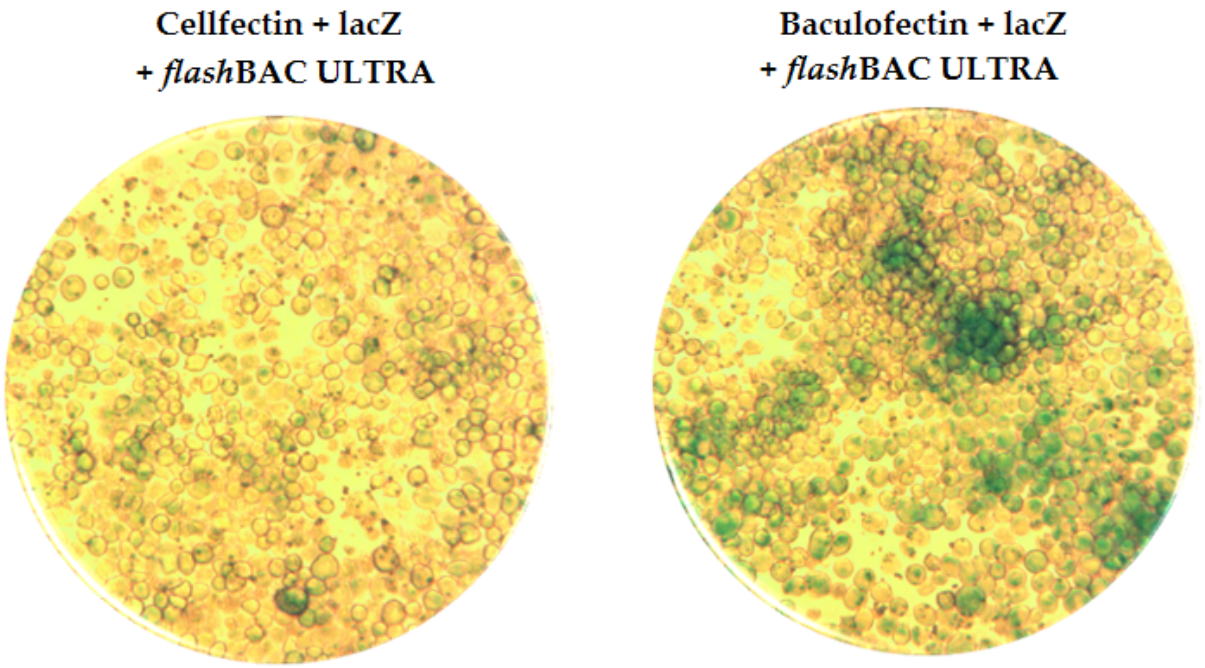

Figure 24: ***Recombinant virus infected cells expressing lacZ (beta-galactosidase).*** *The transfer vector containing a lacZ marker gene (under the control of the polyhedrin gene, polh) was transfected into Sf9 cells together with flashBAC ULTRA DNA using either Cellfectin or Baculofectin II transfection reagent. The recombination of the lacZ gene into the baculovirus genome was analyzed by identifying if X-gal can be metabolized by beta-galactosidase to produce a blue product. According to the proportional blue color development, the recombination had occurred more efficiently in the Baculofectin II transfected cells. Therefore, baculofectin was the preferred transfection reagent in the production of recombinant baculovirus seed stocks (P0).*